The Role of Semaphorin6A in the Formation of Boundary Cap Cells

---

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Olivier Mauti

von

Niedererlinsbach SO

Promotionskomitee

Prof. Dr. Esther Stoeckli (Leitung der Dissertation)
Prof. Dr. Stephan Neuhauss
Prof. Dr. Martin Schwab

Zürich, 2009

INDEX

1. Summary 3
2. Introduction 5
3. Boundary Cap Cells 12
4. Expression Pattern Analysis of Plexins and Neuropilins 25
5. Functional Analysis of Semaphorin6A 42
6. Additional Materials and Methods 61
7. Discussion 76
8. Acknowledgments 83
9. Curriculum Vitae 84
10. References 85

1. Summary

The dorsal neural tube is the origin of neural crest cells which migrate in a stereotypic manner to form a large variety of peripheral nervous system (PNS) structures, such as the dorsal root ganglia (DRGs), the sympathetic ganglia, the enteric nervous system, and the boundary cap cell (BCC) clusters. BCCs are located at the transition zone between central and peripheral nervous system and can be identified by the expression of Krox20, Cadherin-7, protein zero (P_0), and other molecules. Ultrastructural analyses of the trunk spinal cord revealed that BCCs tightly ensheath axons passing the transition zone and that these cells appear ventrally after the outgrowth of motor axons and dorsally before the ingrowth of primary sensory axons. BCCs are transient and disappear postnatally in rats after day 6 (P6). A few studies revealed interesting functions of these cells, mainly acting as "border control" at the transition zone: the dorsal BCCs were shown to be necessary for the ingrowth of primary sensory axons into the spinal cord and to contribute to nociceptive neurons and satellite cells of the DRG. In contrast, ventral BCCs were shown to maintain the integrity of the motor column, since in their absence motor neurons left the spinal cord and migrated along the ventral roots.

In my PhD thesis, I analyzed the function of Semaphorin6A, a transmembrane class-6 Semaphorin, during embryogenesis. At early developmental stages Semaphorin6A shows a very restricted expression pattern in the dorsal neural tube and in the transition zone between CNS and PNS of the lumbar spinal cord of chicken embryos. Its expression overlaps spatio-temporally with Krox20, Cadherin-7, and P_0, indicating that Semaphorin6A is expressed in BCCs. Downregulation of Semaphorin6A using *in ovo* RNAi resulted in emigration of motor neurons along the ventral roots as previously observed after the ablation of BCCs. Additionally, the organization of the dorsal roots was severely disturbed resulting in fused dorsal roots at various levels and irregular DRG shapes. Analysis of BCC localization revealed disorganization or even absence of the BCC clusters.

Previously I had analyzed the expression patterns of the Semaphorin receptors, Plexins and Neuropilins, throughout development. Based on these data, PlexinA1 was a good candidate for the interaction with Semaphorin6A in the lumbar spinal cord due to its strong expression in the lateral motor column. Downregulation of PlexinA1 phenocopied the motor neuron translocations, the disorganization of the dorsal roots, and the malformation of the BCC clusters as seen in the absence of Semaphorin6A function. Based on these results I propose the following working hypothesis for the formation of the ventral BCC clusters: migrating neural crest cells express Semaphorin6A that recognizes PlexinA1 present on motor axons. This interaction stops the migration of neural crest cells resulting in their aggregation and finally the formation of BCC clusters. The absence of either Semaphorin6A on neural crest cells or PlexinA1 on motor axons results in the failure of BCC aggregation, and thus, in loose or even missing BCC clusters. As a consequence, the motor column is destabilized leading to the previously reported motor neuron emigration. The formation of the dorsal BCC clusters differs from the ventral ones due to their appearance at the prospective dorsal root entry site before the ingrowth of sensory axons. Therefore their formation cannot be explained by the same mechanism.

Zusammenfassung

Das dorsale Neuralrohr ist der Ursprung von Neuralleistenzellen, welche entlang von zwei spezifischen Migrationswegen wandern um Bestandteile des peripheren Nervensystems zu bilden. Die Spinalganglien, die sympathischen Ganglien, das enterische Nervensystem, sowie die Boundary Cap Zellen (BCCs) zwischen dem peripheren und dem zentralen Nervensystem sind deren Abkömmlinge. Die BCCs können durch die spezifische Expression von Krox20, Cadherin-7, Protein null (P_0), sowie weiteren Molekülen identifiziert werden. Ultrastrukturelle Analysen des Rückenmarks haben ergeben, dass die BCCs die Axone, welche die Übergangszone passieren, eng umschlingen. Die ventralen BCCs aggregieren erst nachdem die Motoraxone in die Peripherie wachsen, während die dorsalen BCCs vor dem Einwachsen von sensorischen Axonen aggregieren. Die BCCs sind transient und verschwinden in Ratten 6 Tage nach der Geburt. Einige Studien haben die Funktion von BCCs analysiert und herausgefunden, dass sie hauptsächlich eine Kontrollfunktion an der Übergangszone haben: die dorsalen BCCs sind für die Innervation von primären sensorischen Axonen ins Rückenmark von grosser Bedeutung. Im Gegensatz dazu, halten die ventralen BCCs die Integrität der Motorsäule aufrecht. In Abwesenheit der ventralen BCCs verlassen Motorneurone das Rückenmark entlang der Vorderwurzeln.

In der vorliegenden Arbeit habe ich die Funktion von Semaphorin6A, einem transmembranären Klasse 6 Semaphorin, während der Embryogenese analysiert. Während der frühen Embryogenese ist die Expression von Semaphorin6A auf wenige Zellen im Neuralrohr sowie auf die Grenze zwischen zentralem und peripheren Nervensystems in der Beinregion des Hühnerembryos begrenzt. Die Expression überlappt zeitlich und örtlich mit Krox20, Cadherin-7 und P_0, was darauf hinweist, dass Semaphorin6A in den BCCs exprimiert ist. Die Herunterregulierung von Semaphorin6A mittels *in ovo* RNS Interferenz führt zum Auswandern von Motorneuronen, was bereits schon in der Abwesenheit von BCCs beobachtet wurde. Zusätzlich war die Organisation der Hinterwurzeln an unterschiedlichen Orten beeinträchtigt. Die Spinalganglien wiesen zudem unregelmässige Formen auf. Die BCCs waren entweder unorganisiert oder abwesend.

In einer vorangehenden Studie habe ich die Expression von Plexinen und Neuropilinen, beides Rezeptoren für Semaphorin6A, analysiert. Die erhaltenen Expressionsmuster deuteten darauf hin, dass PlexinA1 ein guter Interaktionspartner für Semaphorin6A ist. Die Herunterregulierung von PlexinA1 hatte die gleichen Phenotypen zur Folge, welche in Abwesenheit von Semaphorin6A beobachtet wurden. Aufgrund der erhaltenen Daten postuliere ich folgenden Mechanismus für die Aggregation der BCCs: Migrierende Neuralleistenzellen exprimieren Semaphorin6A welches PlexinA1 auf der Oberfläche von Motoraxonen erkennt. Diese Interaktion führt zu einem Migrationsstopp der Neuralleistenzellen. Dadurch aggregieren sie und formieren sich schlussendlich zu den BCC Clustern. In Abwesenheit von entweder Semaphorin6A oder PlexinA1 ist die Aggregation von BCCs beeinträchtigt, was eine Translokation von Motorneuronen zur Folge hat. Im Unterschied zu den ventralen BCCs, erscheinen die dorsalen BCCs bevor die primären sensorischen Axone ins Rückenmark einwandern. Der beschriebene ventrale Mechanismus kann daher die Aggregation der dorsalen BCCs nicht erklären.

2. Introduction

In the adult nervous system, the transition zone where axons enter or exit the spinal cord, is characterized by the presence of an impenetrable glial barrier formed by the apposition of CNS-derived astrocytes and PNS-derived Schwann cells. However, during embryogenesis when the neuronal circuits are formed, PNS axons have to interconnect with CNS neurons, and motor axons extend out of the CNS into the periphery to innervate target muscles. During that specific period the barrier has to be permissive for axons. The boundary cap cells (BCCs) which are neural crest (NC) cell derivatives were shown to populate the transition zones in order to permit axonal elongation across the embryonic CNS:PNS border (Golding et al., 1997; Golding and Cohen, 1997).

In this chapter, I give a general overview about the establishment of PNS structures. Chapter 3 focuses specifically on the current knowledge about BCCs and genes expressed by them. Chapter 4 describes my detailed analysis of the expression pattern of Plexins and Neuropilins which are known receptors for Semaphorins. These findings are taken as a basis for the functional analysis of Semaphorin6A in the chicken embryo described in chapter 5. The functional analysis of Semaphorin6A and its interaction partner was done using the *in ovo* RNAi technique previously established in our lab (Pekarik et al., 2003) and is summarized in chapter 6. Finally, I discuss the importance and interpretation of my findings in chapter 7.

The neural crest

PNS structures are derived from a pluripotent and transient population of cells located in the dorsal neural tube, the NC cells (Le Douarin and Kalcheim, 1999). During late gastrulation, the NC is induced by a complex interaction of environmental cues acting at the non-neural ectoderm border involving Wnt and fibroblast growth factor (FGF). In the presence of intermediate levels of bone morphogenetic protein (BMP), Pax3/7, Zic1, and Msx1/2 are induced which act synergistically in the presence of Wnt to upregulate NC specifiers in the dorsal neural tube, such as FoxD3, Sox9, and Snail. The NC progenitor pool is kept in a multipotent and proliferative state through interaction of the proto-oncogene c-Myc and the basic Helix-Loop-Helix (bHLH) gene Id3 (Light et al., 2005), while cell death is prevented via Sox9 and Snail1/2 (Cheung et al., 2005). A complex interaction between Snail, Sox9, and FoxD3 induces the epithelial to mesenchymal transition (EMT) which is characterized by a loss of apical-basal cell polarity, a switch from tight to gap junctions, and downregulation of various cell adhesion molecules (reviewed in Thiery and Sleeman, 2006). The switch from epithelial cell specific type I N-Cadherin to type II Cadherin-7 results in a decreased NC cell adhesiveness (see below; Chu et al., 2006). The subsequent NC cell delamination is facilitated by a reduction of Laminin at the basal surface of the dorsal neural tube, most likely by proteolytic activity of NC cells, and upregulation of Integrin-β1 (Valinsky and Le Douarin, 1985; Cheung et al., 2005). Delaminating and subsequently migrating NC cells maintain their pluripotency by Sox10, which also prevents premature neuronal differentiation (Kim et al., 2003).

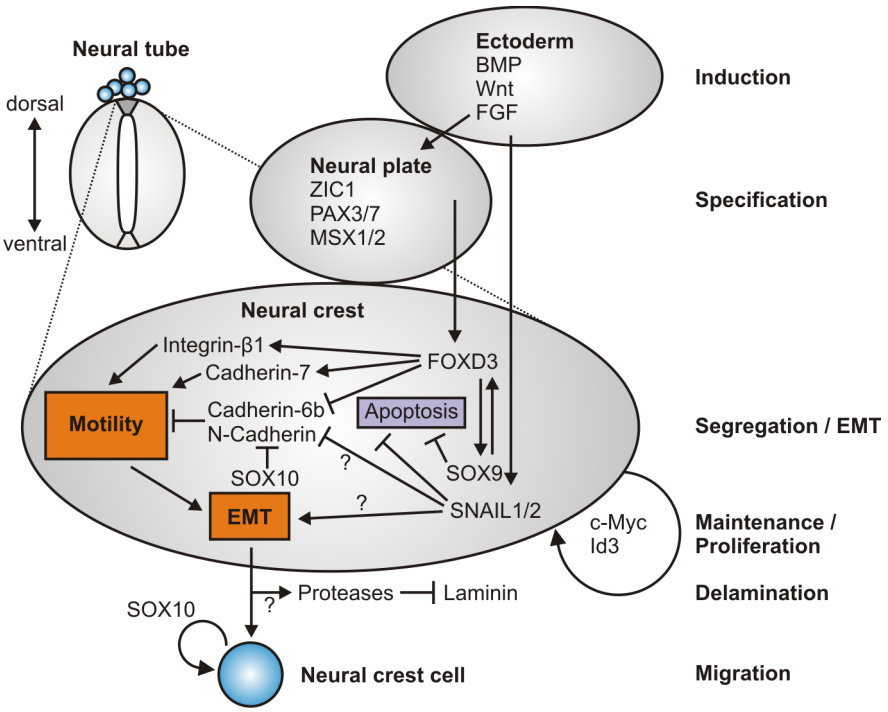


Figure 1: Induction and delamination of neural crest cells.

The concerted interaction of BMP, Wnt, and FGF at the non-neuronal ectoderm induces the neural plate during late gastrulation. In the presence of Wnt, NC-specifiers are upregulated that in turn induce the maturation of NC cell progenitors. Finally, NC cells undergo an epithelial to mesenchymal transition (EMT) and delaminate from the neural tube. (Modified from Thiery and Sleeman, 2006)

NC cells of mice lacking Pax3 (*Splotch*; Serbedzija and McMahon, 1997), SoxE (Southard-Smith et al., 1998; Cheung et al., 2005), or Slug/Snail (Jiang et al., 1998) fail to delaminate or undergo apoptosis resulting in defects in PNS structures or even their absence. Similarly, surgical ablation of the dorsal neural tube results in a loss of NC cells, and thus, in missing PNS structures (Vermeren et al., 2003).

The extracellular matrix

The extracellular matrix (ECM) is a tight network of a large number of structural proteins giving unique flexible and elastic properties to many tissues, e.g. the skin or the sclerotome. During embryogenesis, the ECM plays an integral role in the formation of the nervous system by providing an appropriate surrounding and support to migrating NC cells and axons. The structure of the ECM is primarily formed by proteins that belong to the family of Collagens and Elastins (Duband and Thiery, 1987). While Collagen fibers help to stabilize the ECM, Elastin fibers provide elasticity and both together are important for maintaining the unique ECM structure. Cross-links between the main fibers of the ECM are generated by Fibronectin and Laminin, and more importantly, they serve as adhesive substrates for migrating cells and growing axons (Newgreen and Thiery, 1980; Bixby and Harris, 1991; Tucker and McKay, 1991). Other components of the ECM are Tenascins, Aggrecan, Versicans, Thrombospondin-repeat containing proteins (e.g. F-Spondin) that act mainly as repellents for migrating NC cells and axons (Bixby and Harris, 1991; Perris, 1997; Debby-Brafman et al., 1999). The aqueous environment of the ECM is maintained by Heparan Sulfate Proteoglycans (HSPG) which form a gel-like mixture (reviewed in Iozzo, 1998). Furthermore, HSPGs have the ability to trap growth factors and help to distribute morphogens across the ECM, such as Sonic Hedgehog which is secreted from the notochord (Guerrero and Chiang, 2007). The balance of all these factors defines and influences the permissiveness of the ECM for cell motility and axon guidance.

Neural crest cell migration

The vertebrate embryo has a segmental organization and each segment, the so-called somite, is subdivided into anterior and posterior sclerotome. The delamination of NC cells takes place all along the anterior-posterior axis of the embryo but their migration is restricted to the anterior sclerotome due to the non-permissive posterior sclerotome (Keynes and Stern, 1984; Kalcheim and Teillet, 1989). In the trunk of vertebrates, several migratory waves occur that form different NC derivatives during embryogenesis. NC cells migrate in a stereotypic manner from their origin in the dorsal neural tube to their prospective target sites where they condensate and form the PNS structures. The majority of migrating NC cells can be traced by their specific expression of Sox10, the HNK-1 and the 1E8 antigens (Tucker et al., 1984; Bhattacharyya et al., 1991; Kuhlbrodt et al., 1998). During NC cell migration various repulsive guidance molecules in the ECM form permissive and non-permissive paths; examples are F-Spondin, secreted class 3 Semaphorins, and Ephrins (Bronner-Fraser, 1994; Krull et al., 1997; Debby-Brafman et al., 1999; Eickholt et al., 1999). They are found in the posterior

sclerotome, the perinotochordal area, and (at early developmental stages) in the dermomyotome which are normally avoided by migrating NC cells. ECM proteins such as Laminin and Fibronectin are necessary substrates and perturbing their docking sites using function-blocking antibodies strongly interferes with NC cell migration (Boucaut et al., 1984). Integrin-β1, a necessary component of the receptor for Laminin and Fibronectin, is playing a major role in facilitating NC cell migration (Horwitz et al., 1985; Lallier et al., 1994). The conditional deletion of Integrin-β1 in NC cells of mice showed severe defects in the formation of the PNS, mainly resulting from a delayed migration of NC cells (Pietri et al., 2004). The molecular mechanism underlying NC cell migration is not fully understood, but the extension of NC cell filopodia is comparable to the motility of the growth cone: at the surface of filopodia different receptors are expressed that transduce environmental cues into precise guidance responses. The extension or retraction of NC cell filopodia requires active actin cytoskeleton dynamics. One of the molecules implicated in actin filament dynamics belongs to the Ena/VASP family (Drees and Gertler, 2008). Their function is to antagonize the capping protein that prevents further polymerization of the actin filaments (Bear et al., 2002). Two recent studies discuss this protein family as being downstream of repulsive receptors during NC cell migration: Ena/VASP proteins mediate repulsion from Ephrin ligands (Evans et al., 2007) and from Sema6D during endocardiac crest cell migration (Toyofuku et al., 2004).

Pathways of neural crest cell migration

Early migrating NC cells travel ventrally in the anterior sclerotome between the neural tube and the dermomyotome and give rise to most PNS structures like DRGs, sympathetic ganglia, enteric nervous system, Schwann cells, and BCCs (Figure 2; Rickmann et al., 1985; Bronner-Fraser, 1986; Le Douarin et al., 1992; Niederländer and Lumsden, 1996). Conversely, the majority of late delaminating NC cells travel dorsolaterally, i.e. between the ectoderm and the dermomyotome, to form melanocytes of the skin, cartilage derivatives, and smooth muscles (Erickson et al., 1992; Le Douarin et al., 1992; Bronner-Fraser, 1994).

For a long time the migratory pattern of NC cells was believed to be topographically defined in the NC, since apically located NC cells migrate along the dorsolateral pathway and basally positioned cells travel along the ventromedial pathway. However, different studies showed that a combination of environmental cues and their time point of delamination define their respective pathways: all early delaminating NC cells migrate along the ventromedial pathway in the anterior sclerotome. Eph-Ephrin and Sema3A-Plexin/Neuropilin interactions were shown to restrict the migration of NC cells to the anterior sclerotome (Krull et al., 1997; Eickholt et al., 1999). Late migrating NC cells travel in a non-segmented manner dorsolaterally between the epidermis and the dermomyotome. The decision point for the dorsolateral or ventromedial migration appears to be the dorsal edge of the dermomyotome (Figure 2B). Recently, Jia and coworkers discovered Robo/Slit signaling that guides late emigrating NC cells to the dorsolateral pathway (Jia et al., 2005): Robo-1 is expressed in early migrating NC cells and its ligand Slit2 was shown to repel NC cells. Slit2 is expressed in the dermomyotome and thus, restricts early NC cells to the ventromedial pathway. F-Spondin which was shown to repel NC cells is

downregulated in the dermomyotome when NC cells are about to migrate dorsolaterally (Debby-Brafman et al., 1999). The distribution of other molecules in the ECM, e.g. Versicans, forms various non-permissive regions in the sclerotome for migrating NC cells (Landolt et al., 1995; Dutt et al., 2006). There is also evidence for attractive cues that positively influence the migration of NC cells through the anterior sclerotome (Krull, 2001). In conclusion, the combination of various molecules present in the sclerotome guides migrating NC cells to their prospective targets. The knockout of either of these genes does not disturb the migration pattern, suggesting that NC migration is controlled by a multitude of redundant positive and negative factors (Wang et al., 1998; Kawasaki et al., 2002).

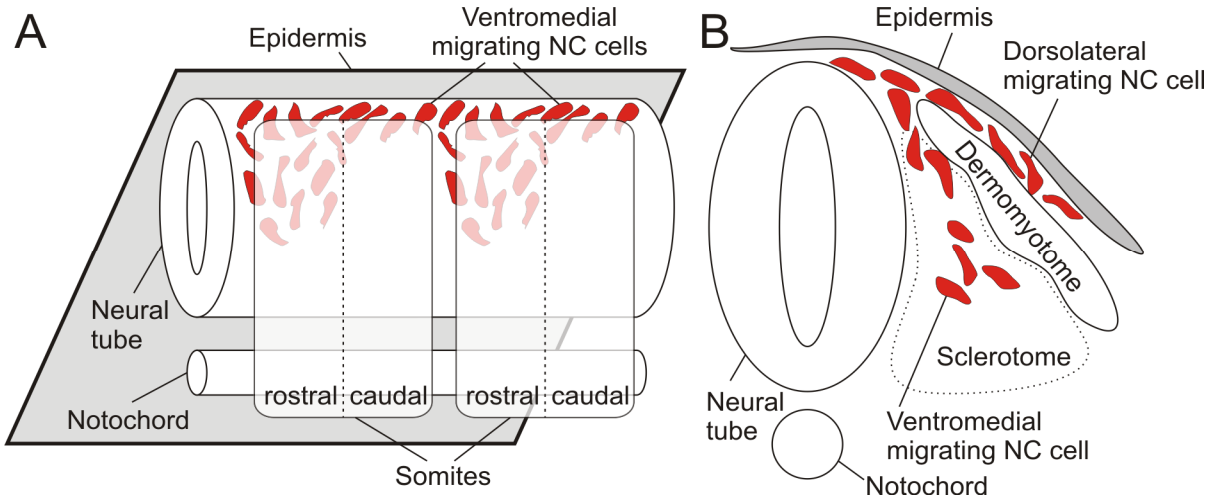


Figure 2: Migration of NC cells along the dorsoventral axis of the embryo.
(A) NC cells delaminate at all rostrocaudal levels of the embryo. Their ventromedial migration pathway is restricted to the anterior (rostral) somite by a non-permissive barrier present in the posterior (caudal) sclerotome. (B) NC cells migrating along the ventromedial pathway, i.e. between the neural tube and the dermomyotome, give rise to PNS structures, while dorsolaterally migrating NC cells travel between the dermomyotome and the epidermis and form mainly pigment cells. (Modified from Halloran and Berndt, 2003)

Dorsal root ganglia

One of the most prominent structures of the PNS are the DRGs located in the anterior sclerotome of each somite. The DRGs contain the soma of bipolar sensory neurons that are NC cell derivatives. They differentiate into the sensory lineage under control of Sox10 and the bHLH transcription factors Neurogenin-1 and -2 (reviewed in Marmigere and Ernfors, 2007). Two major successive but overlapping waves of NC cell migration contribute to DRG neurogenesis and form three distinct neuronal subclasses according to target innervation, structural, and biochemical properties. Cells of the early wave are Neurogenin-2 positive and mainly give rise to mechano- and proprioceptive neurons expressing the neurotrophin receptors TrkB and/or TrkC (Ma et al., 1999; Rifkin et al., 2000). Mechanoceptive neurons innervate large areas of the skin and transmit innocuous (touch) stimuli, while proprioceptors innervate muscles and transmit positional information. NC cells of the late migratory wave contribute mostly to TrkA-positive nociceptors under the control of Neurogenin-1 and mediate noxious (pain) stimuli. In a recent study, George and coworkers reported that the late wave of NC cell migration originates from an ipsi- and a contralateral pool of the NC (George et al., 2007). The

ipsilateral NC cells populate the inner DRG core and become neurons while the contralateral NC cells cross the dorsal midline and colonize the dorsal roots and dorsal parts of DRG. Preventing the migration of the contralaterally emigrating NC cells resulted in a 50% reduction of TrkA-positive neurons. Another source of TrkA-positive neurons appears to be the BCCs: a third wave of NC cell migration was reported originating from the BCCs (Maro et al., 2004). Fate-mapping the progeny of BCCs revealed that up to 5% of TrkA-positive DRG neurons and peripheral glia are derived from BCCs. Surprisingly, in the absence of BCCs approximately 50% of TrkA-positive neurons were absent in the DRGs. How can this difference be explained? A possibility would be that BCC-derived cells either induce the differentiation of other nociceptive progenitors located in the DRG perimeter and/or the lack of BCC-derived glial cells results in the atrophy of other TrkA-positive neurons. However, it is unknown if BCC-derived glial cells preferentially associate with TrkA-positive nociceptors. In summary, all TrkA-positive neurons originate directly and indirectly from the second NC cell migration wave, some of which derive from a contralateral pool of the NC and some derive from BCCs.

Shortly after the formation of the sensory neurons, the first axons start to elongate in order to interconnect with other neurons in the spinal cord and to innervate sensory targets in the periphery. The initial pathway of sensory axons along the dorsoventral axis is supposed to be shaped by surround repulsion rather than by attraction from intermediate targets. The overlying dermomyotome and the notochord (see Figure 2) were shown to repel sensory axons *in vitro* (Keynes et al., 1997). Once the sensory axons reach the dorsal root entry site (DRES) they innervate the spinal cord, where they connect to distinct laminae: nociceptive neurons terminate in the dorsal horn, mechanoreceptors in the deeper laminae, and proprioceptive neurons in the intermediate zone and the motor column of the spinal cord.

Motor neurons

Motor neurons are located in the ventral neural tube and are distinct from interneurons because they project axons into the periphery. They derive from a highly proliferative pool of motor neuron progenitors (pMN). This domain is specified by a combinatorial interaction of various homeodomain proteins induced by the floor plate-derived morphogen Sonic Hedgehog. The earliest determinant of motor neuron identity is the homeodomain protein MNR2 (Tanabe et al., 1998). In turn, MNR2 induces the expression of downstream transcription factors such as Islet-1/2, Lim3, and HB9 that further define motor neuron subpools within the motor column. The postmitotic neurons form rostrocaudally oriented motor columns in the ventral spinal cord based on cell body position, axonal projections, and gene expression (Figure 3). Roughly, the columns can be subdivided into medial motor column (MMC) and lateral motor column (LMC). In comparison to the MMC, which is present all along the rostrocaudal axis of the spinal cord, the LMC is only present at lumbar and brachial levels. Motor neurons of the MMC project into epaxial (back) and hypaxial (body wall and limb) muscles. In contrast, LMC neurons innervate muscles of the limbs and can be further divided into medial LMC (LMC_M) and lateral LMC (LMC_L). In retrograde labeling experiments of limb muscles, neurons of the LMC_M and LMC_L were shown to innervate ventral and dorsal limb muscles, respectively (Landmesser,

1978). Shortly after the migration of motor neuron cell bodies to their final locations in the ventral neural tube, they start to send out axons. At that time point the basal lamina surrounding the spinal cord is not dense and motor axons were shown to penetrate the basal lamina at the ventral motor axon exit point (VMEP) (Fraher et al., 2007). They further bundle to form the ventral roots and associate with sensory axons in the plexus region. Finally, they separate again in dorsal and ventral nerves which innervate the corresponding limb muscles.

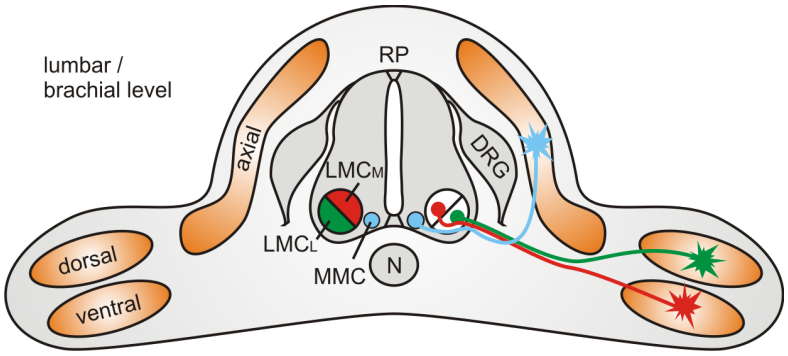


Figure 3: Organization of motor neurons and their prospective targets during embryogenesis.
Motor neurons are formed from a proliferating progenitor pool located in the ventral ventricular zone. Postmitotic motor neuron precursors migrate to their prospective locations in the ventral spinal cord where they form rostrocaudal motor columns. The LMC is only present at limb levels, while the MMC extends all along the anterior-posterior axis. The LMC can be further divided into subpools innervating the dorsal (LMC_L) and ventral (LMC_M) limb muscles. The MMC innervates axial (body wall and back) muscles. (Modified from Jessell, 2000)

3. Boundary Cap Cells

Summary

The boundary at the interface between the central and the peripheral nervous system consists of a glial cell barrier. In adult vertebrates, the barrier is formed by the apposition of CNS-derived astrocytes and PNS-derived Schwann cells which is impenetrable for regenerating axons. However, during embryogenesis sensory and motor axons have to pass the transition zone in order to connect to prospective targets in both, the CNS and in the periphery. The embryonic glial barrier differs from the adult one in terms of axonal permissiveness. A transient NC-derived population of cells, the so-called boundary cap cells (BCCs), occupy the transition zones and act as a gatekeeper. The BCCs allow axons to enter and exit the spinal cord; they control the maturation of the adult glial barrier and preserve the cellular integrity of the central and the peripheral nervous system. Furthermore, BCCs were shown to contribute to the formation of other PNS structures, such as the DRG neurons and glial cells. In the absence of BCCs the integrity of both the central and the peripheral nervous system is seriously compromised resulting in the intermixing of CNS and PNS cells and loss of neurons. The present chapter summarizes the different functions of BCCs and discusses BCC-specific genes.

BCCs populate the transition zones

The entry and exit points of the embryonic transition zone between the central and the peripheral nervous system are characterized by the presence of a highly proliferating and transient cell population that derives from the neural crest (NC) and are formed during a late wave of NC cell migration (Altman and Bayer, 1982; Altman and Bayer, 1984; Niederländer and Lumsden, 1996; Golding and Cohen, 1997). These cells, the so-called boundary cap cells (BCCs), were identified in rodents and birds (Niederländer and Lumsden, 1996; Golding and Cohen, 1997). In rats, they appear by E13 and persist until postnatal day 6 (P6) (Altman and Bayer, 1984; Golding and Cohen, 1997). In chicken embryos, they appear in the lumbosacral spinal cord at Hamburger-Hamilton stage 18 (HH18; Hamburger and Hamilton, 1951) and persist at least until HH36 (Mauti et al., 2007). In mice they were shown to populate the transition zones between E10.5 and were found until after birth (Baron-Van Evercooren et al., 2008).

In the trunk spinal cord, two distinct BCC clusters are formed, the ventral clusters located at the ventral motor axon exit points (VMEP), where motor axons leave the spinal cord, and the dorsal clusters located at the dorsal root entry zone (DREZ), where the primary sensory afferents enter the spinal cord (Figure 4). The cervical spinal cord consists of a VMEP and a lateral axon exit and entry point (LEP) that is shared by spinal accessory motor and sensory axons and both are occupied by BCCs (Snider and Palavali, 1990). In contrast, only one common entry and exit point containing BCCs is formed in the hindbrain (Niederländer and Lumsden, 1996).

BCCs express a diversity of genes, most of which exhibit unknown functions in this context (for details, see below): Krox20, Cadherin-7, Lingo-1, Sox10, SSEA-1, Erythropoietin (EPO), Monoamine

oxidase B (MAOB), neurotrophin receptor TrkB, protein zero (P_0), chemokine receptor Cxcr4, Lgi4, Semaphorin3B, -3G, -6A, and others (Wilkinson et al., 1989; Bhattacharyya et al., 1991; Ernfors et al., 1992; Nakagawa and Takeichi, 1995; Golding and Cohen, 1997; Vitalis et al., 2003; Belmadani et al., 2005; Hjerling-Leffler et al., 2005; Knabe et al., 2005; Okafuji and Tanaka, 2005; Bermingham et al., 2006; Bron et al., 2007; Mauti et al., 2007).

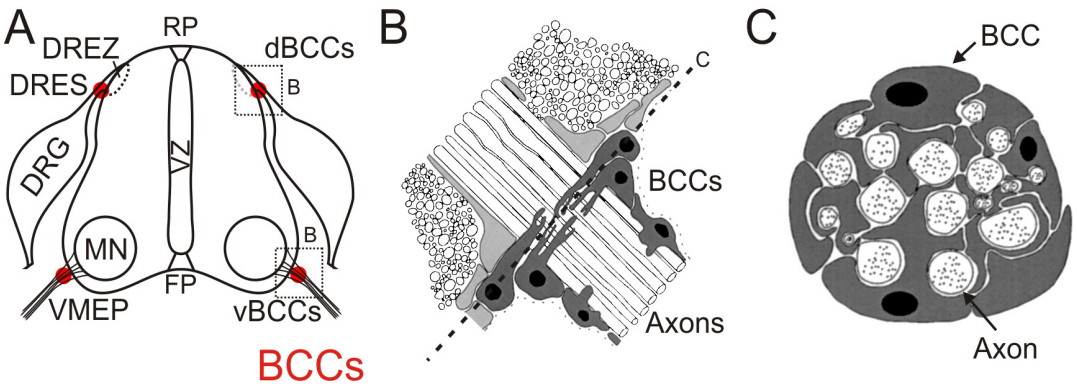


Figure 4: Boundary cap cells in the trunk spinal cord.
(A) The roof plate (RP) is the origin of neural crest cells that migrate along the ventromedial pathway to form dorsal root ganglia (DRGs) and boundary cap cells (BCCs). DRG neurons extend their neurites, form the dorsal roots, and cross the dorsal BCCs (dBCCs) at the dorsal root entry site (DRES) in order to innervate the spinal cord at the dorsal root entry zone (DREZ). The motor neurons (MN) extend their axons ventrolaterally and exit the spinal cord at the ventral motor axon exit point (VMEP). The VMEPs are populated by the ventral BCCs (vBCCs). (B) Axons passing the CNS:PNS boundary are covered by BCCs and ensheath each single axon at the transition zone as seen in (C). (Adapted from Fraher, 1997)

Aggregation of BCCs

For a long time, the BCCs were thought to delineate the prospective entry and exit point at the border of the CNS based on the observation that they appeared at the transition sites before axonal contact (Niederländer and Lumsden, 1996; Golding and Cohen, 1997; Vermeren et al., 2003). However, a detailed ultrastructural study during the formation of VMEP and DRES revealed for the first time that the ventral BCCs appear after motor axons exit the spinal cord at all spinal and cranial levels (Fraher et al., 2007). In contrast, the dorsal BCCs appear before the ingrowth of primary sensory afferents. The mechanism that arrests NC cells at the prospective dorsal or ventral transition zone remained unknown. A favorite candidate mediating this function was Cadherin-7 (Cad-7), a Ca^{2+}-dependent homophilic cell adhesion molecule. It is expressed in migrating NC cells and later in BCCs (see below; Nakagawa and Takeichi, 1995). However, no Cad-7 expression was found at the prospective BCC locations at the time point of aggregation that could define their prospective locations, i.e. on the neuroepithelium or in the ECM (Golding and Cohen, 1997). Although motor neurons express Cad-7, the onset of expression is after the formation of the ventral BCC clusters, and therefore, Cad-7 is unlikely to be part of the migration arrest mechanism (Nakagawa and Takeichi, 1995). Rather the interaction among Cad-7-positive NC cells and motor axons in the periphery is supposed to be necessary for the aggregation of Schwann cells that populate the peripheral motor axons (Nakagawa and Takeichi, 1995). Thus, the mechanism responsible for the aggregation of BCC remains elusive.

Why are dorsal but not ventral BCCs prefiguring the transition sites?

Initially, BCCs were thought to guide axons to the entry and exit points. However, the possibility that the dorsal BCCs are a potential source of chemoattractants guiding primary sensory afferents to the DREZ could not be confirmed (Keynes et al., 1997). Furthermore, sensory axons of embryos lacking BCCs (knock-in of diphtheria toxin in the Krox20 locus; Krox20-DT) did not fail to grow towards the prospective DREZ (Vermeren et al., 2003; Maro et al., 2004). Albeit the entry site into the spinal cord was normal, the dorsal roots were shorter and often defasciculated. Keynes and coworkers suggested that surround repulsion derived from the overlying dermomyotome and the notochord provides a mechanism that guides sensory afferents to the dorsal neural tube (Keynes et al., 1997).
An interesting finding was that the basal lamina of the neural tube underlying the dorsal BCCs disappeared shortly after their aggregation but before axonal contact (Niederländer and Lumsden, 1996; Fraher et al., 2007). Since the basal lamina of the dorsal neural tube displays an impenetrable barrier for sensory axons, it is likely that the preoccupation of the DREZ by BCCs is necessary to prepare the ingrowth for sensory axon. Evidence is provided by different studies that discovered secretion of proteases and plasminogen activators from NC cells which are necessary for their migration through the ECM and invasion of different tissues (Figure 5; Valinsky and Le Douarin, 1985; Erickson et al., 1992; Carroll et al., 1994). The disappearance of the basal lamina shortly before the first NC cells emerge from the dorsal neural tube further confirmed this observation (Cheung et al., 2005).
In contrast, the basal lamina covering the ventral neural tube is not fully established when the first motor axons exit the spinal cord. Motor axons were shown to penetrate the basal lamina. The basal lamina does not seem to be degraded by this process because fragments can be detected on the distal part of the motor axon (Fraher et al., 2007). In agreement with this observation, motor axon outgrowth and location was not perturbed in the absence of ventral BCCs (Vermeren et al., 2003).

Dorsal BCCs control the maturation of the DREZ

Several studies performed during the past decades addressed the function of BCCs. In 1997, Golding and coworkers investigated the function of dorsal BCCs (Golding et al., 1997; Golding and Cohen, 1997). In this study the authors propose two key aspects of dorsal BCCs: (1) they control and promote the initial ingrowth of primary sensory afferents into the spinal cord, and (2) they are involved in the maturation of the DREZ after birth. In cryoculture experiments they could show that embryonic sensory neurites preferentially grew through dorsal BCCs while P6 sensory neurons did not. In a second experiment they found that E18 and P6 neurons could extend neurites equally well when they were in contact with CNS tissue, suggesting that the failure of neurite extension into the P6 spinal cord is not due to growth-inhibitory molecules expressed in the CNS. Surprisingly, embryonic DRG neurons were able to extend neurites and grow through the mature P6 DREZ, concluding that the failure of P6 DRG neurites to innervate the CNS is based on the expression of receptors recognizing the inhibitory ligands present in the mature (P6) DREZ. The authors found that the maturation of the

DREZ involves the invasion of astrocytes into the dorsal roots. Astrocytes were reported to express Tenascins which are able to repel sensory axons *in vitro* (Pindzola et al., 1993; Taylor et al., 1993). Because BCCs disappear upon maturation of the DREZ, the authors concluded that dorsal BCCs prevent the premature invasion of astrocytes into the DREZ. Conversely, astrocytes were shown to prevent the invasion of Schwann cells into the CNS (Sims et al., 1985).

BCCs contribute to PNS structures

BCCs were shown to proliferate extensively during embryogenesis. Still, the size of BCC clusters decreased from E17 onwards and they completely disappeared by P6 (Golding and Cohen, 1997). The decrease in size cannot be explained by apoptosis since no increase in TUNEL-positive BCCs was detected beyond E16 (Altman and Bayer, 1984; Golding and Cohen, 1997). In 2004, Maro and coworkers found a possible explanation for the loss of BCCs (Maro et al., 2004). Crossing $Egr2^{Cre}$ (Krox20) mice with reporter mice in which β-galactosidase was activated upon Cre-mediated recombination allowed them to trace the progeny of BCCs. Soon after E11, i.e. when the BCCs have settled down, many β-galactosidase positive cells were found in the dorsal and ventral roots and in the DRGs. Colabeling of β-galactosidase-positive cells with ErbB3, a marker for PNS glial cells and uncommitted NC precursors, suggested that all Schwann cells of the dorsal roots were BCC-derived. Furthermore, β-galactosidase-positive cells left the ventral BCCs and populated the ventral roots and the ventral pole of the DRGs (Figure 6). However, the authors could not investigate if the entire glia cell population of the PNS were BCC-derivatives since Krox20 was upregulated in all Schwann cells after E15.5. The majority of β-galactosidase-positive cells in the DRG was either TrkA positive, a marker for nociceptive neurons, or surrounded TrkA-positive cells, suggesting that they could be glial satellite cells. Why do BCCs mainly contribute to nociceptors and Schwann cells? NC cells of the two major migratory waves occurring during embryogenesis differ in the expression of Neurogenins (Ma et al., 1999). NC cells migrating during the early wave are Neurogenin-2 positive and mainly give rise to TrkB- and TrkC-positive neurons while the late NC cells are Neurogenin-1 positive and generate primarily TrkA-positive neurons and BCCs. It is therefore likely that the progeny of BCCs are biased toward a nociceptive lineage, and thus, mainly gives rise to TrkA-positive neurons. Interestingly, fate mapping of glia cells in the dorsal roots showed that they derive from Neurogenin-2-positive precursors (Zirlinger et al., 2002) which is in conflict with the proposed origin of BCCs from the late, Neurogenin-1-positive wave. A possibility would be that BCCs can create different sensory lineages that differ in their Neurogenin expression. Evidence for that was found in two recent studies: BCCs were shown to possess stem cell-like characteristics and to be able to differentiate into multiple sensory subtypes and glia cells (Hjerling-Leffler et al., 2005). In a recent study, BCCs were even shown to differentiate into mature Schwann cells, both *in vivo* and *in vitro* (Aquino et al., 2006).

In summary, BCCs control the maturation of the DREZ and contribute largely to nociceptive neurons, glial satellite cells of the DRGs, and to all Schwann cells of the dorsal roots.

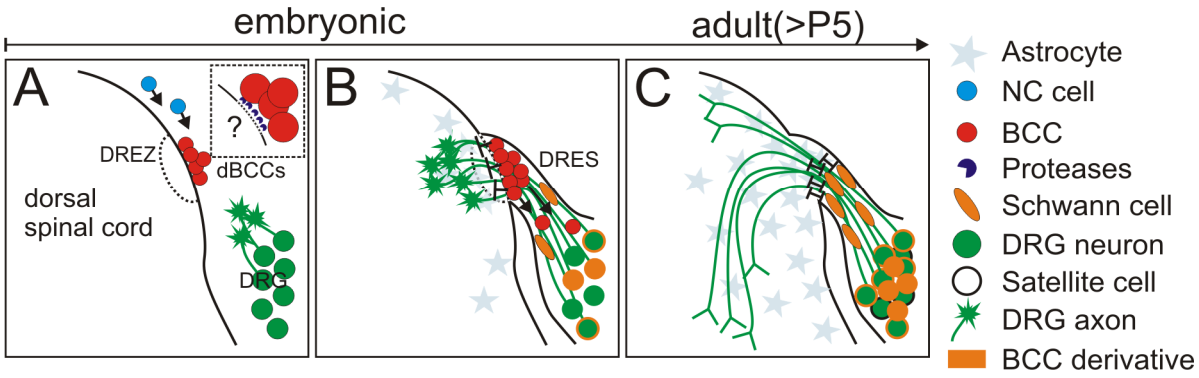


Figure 5: Organization and maturation of the dorsal root entry site.
(A) Dorsal BCCs (dBCCs) prefigure the DREZ before the ingrowth of primary sensory axons into the CNS. Inset: dBCCs are thought to degrade the basal lamina by the secretion of proteases and plasminogen activators. (B) dBCCs prevent the invasion of astrocytes into the DRES and possibly control astrocytogenesis via erythropoietin (see below). dBCCs migrate away to give rise to Schwann cells ensheathing the dorsal roots, to nociceptive neurons, and to satellite glia cells of the DRG. (C) Postnatally, the dBCCs disappear and the mature CNS:PNS interface consisting of an apposition of CNS-derived astrocytes and PNS-derived Schwann cells is formed.

Ventral BCCs prevent the emigration of motor neurons

To assess the function of ventral BCCs, Vermeren and colleagues used three experimental models: *Splotch* mice which fail to form BCCs due to missing NC cells in the trunk, a targeted genetic ablation of BCCs using the Krox20-DT mouse (see above), and surgical ablation of the dorsal neural tube resulting in the absence of NC cells (Vermeren et al., 2003). In all three experiments, the absence of BCCs led to a destabilization of the motor column resulting in the emigration of motor neuron somas out of the CNS into the periphery along the ventral roots. However, the phenotype was more prominent in *Splotch* mice and in chicken embryos after dorsal neural tube ablation compared to Krox20-DT transgenic mice. One possibility could be that the death of BCCs happens progressively and the short contact between motor axons and the “intact” BCCs is sufficient to partially stabilize some motor neurons. Another explanation would be that the BCC pool is not homogenously expressing Krox20 and therefore some Krox20-negative BCCs could survive that partially maintain motor column integrity. It is even conceivable that BCCs stabilize only one part of the motor column. In Sox10 mutant mice that fail to form BCCs (see below) ectopic motor neurons were also observed (Bron et al., 2007). Vermeren and coworkers further confirmed the necessity of BCCs in maintaining motor column integrity by grafting quail NC cells into the spinal cord of dorsal neural tube ablated chicken embryos (Vermeren et al., 2003). The NC cell grafts migrated to the VMEP and appeared to form BCCs which prevented motor neuron emigration. In embryos lacking BCCs the ventral roots appeared much thicker and defasciculated. However, whether this is due to motor neuron somas occupying more space or the failure of motor axons to fasciculate remains unknown. The study did not address the organization of the DRG and DREZ. Maro and coworkers could not find misplaced sensory neurons in the proximity of the DRG in mouse embryos lacking BCCs (Krox20-DT; Maro et al., 2004).

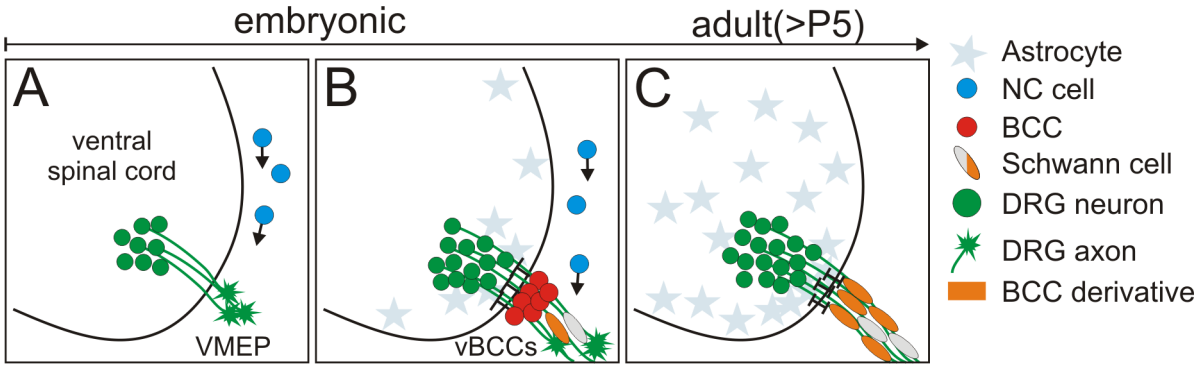


Figure 6: Formation of the ventral BCC clusters.

(A) Motor axons emerge from the spinal cord before the presence of ventral BCCs (vBCCs). (B) Shortly after, vBCCs start to aggregate around the motor axons at the VMEP. The proliferating vBCCs migrate along the ventral roots where they differentiate into Schwann cells (orange oval). Migrating NC cells give also rise to Schwann cells (grey oval). (C) Postnatally, the vBCCs disappear and the transition zone is formed by the direct apposition of astrocytes and Schwann cells.

Genes expressed in boundary cap cells

A number of genes were shown to be expressed in BCCs. In the majority of cases, their function in the context of BCCs is unknown. The following paragraph summarizes genes identified either in BCCs or in NC cells giving rise to BCCs (see also Table 1). Some of the genes play important roles during neurogenesis and mouse mutants lacking these genes often suffer from severe peripheral neuropathies.

Cadherin-7 and N-Cadherin

Cadherins form a large group of Ca^{2+}-dependent cell adhesion molecules that have mostly been implicated in cell-cell interactions and cell sorting (Nakagawa and Takeichi, 1995). They comprise an extracellular, a transmembrane, and a highly conserved cytoplasmic domain. Two types of Cadherins are distinguished based on sequence characteristics (type I and II) (Takeichi, 1995). Type I Cadherins include E-Cadherin (Cdh1, L-CAM), N-Cadherin (Cdh2), P-Cadherin (Cdh3), R-Cadherin (Cdh4), and U-Cadherin, while type II Cadherins comprise cad5-cad12 (Cdh5-12) (reviewed in Pla et al., 2001). Cadherins mediate cell-cell adhesion by homophilic interaction and are anchored to the cytoskeleton via Catenins that link the cytoplasmic domain of Cadherins to actin filaments (reviewed in Kemler, 1993). Generally, premature NC cells express type I but not type II Cadherins. Upon delamination there is a switch from type I to type II Cadherins that is supposed to be important for the ability to migrate, since the adherence of type II Cadherins is much lower than that of type I Cadherins (Chu et al., 2006). Once NC cells home in at their prospective target sites, they switch back to type I Cadherins. However, there are exceptions to this rule as observed for Cadherin-7 in BCCs (see below; Nakagawa and Takeichi, 1995).

During the delamination of NC cells from the dorsal neural tube Cadherin-7 is upregulated in a subpopulation of NC cells migrating ventrolaterally (Nakagawa and Takeichi, 1995; Takeichi, 1995; Nakagawa and Takeichi, 1998). It is not clear, if NC cells differentiating to BCCs already express

Cadherin-7 or if its expression is upregulated once the BCC clusters are formed. However, the function of Cadherin-7 in migrating NC cells is supposed to ensure a coherent migration through the ECM rather than to be required for homing in at specific loci (Nakagawa and Takeichi, 1995; Golding et al., 1997).

Several studies showed a major role for N-Cadherin in cell adhesion, axon growth and guidance. N-Cadherin is expressed in the ventricular zone of the neural tube, in premigratory NC cells, in DRGs, Schwann cells, and BCCs (Wanner et al., 2006). Upon arrest of NC cells at the prospective entry and exit points of the neural tube, the switch from type II to type I Cadherin involves the upregulation of N-Cadherin. N-Cadherin is a valuable candidate mediating cell-cell contacts between BCCs due to its strong adhesive properties. Furthermore, N-Cadherin was reported to be a potent growth-promoting molecule inducing neurite extension in cultured chick ciliary ganglion neurons (Bixby and Zhang, 1990).

Cxcr4

The chemokine receptor Cxcr4 and its ligand stromal cell-derived factor 1 (Sdf1) are mainly known for their function in the hematopoietic stem cell system. However, there is growing evidence that the interaction between Cxcr4 and Sdf1 directs chemotactic cell migration in various developmental processes. The migration of the posterior lateral line (PLL) primordium in zebrafish was reported to depend on the distribution of Sdf1 along the migratory path which is recognized by Cxcr4 on the leading tip of the primordium (David et al., 2002). Similarly, Cxcr4 is expressed in migrating NC cells while Sdf1 is present in the sclerotome, prominent in the region of the prospective DRGs and around the ventral neural tube, suggesting that it could direct the migration of NC cells to their prospective condensation points. Cxcr4 is also expressed in DRGs and motor neurons and seems to be present in BCCs and Schwann cells (Belmadani et al., 2005). Additionally, Sdf1 transcripts were found in the DRGs and dorsal roots (Lieberam et al., 2005; Odemis et al., 2005).

Egr2 (Krox20)

Krox20 is a well established marker for BCCs. It belongs to the group of zinc-finger transcription factors. It is upregulated in a feed-forward loop involving Brn2 and Sox10. Krox20 was reported to control the segmentation of the hindbrain and the myelination of the PNS (Schneider-Maunoury et al., 1993; Topilko et al., 1994). In mice, the onset of Krox20 expression was detected by radioactive in situ hybridization at E8.5 in the dorsal neural tube, possibly in precursors of BCCs (Wilkinson et al., 1989). However, in the avian embryo, no Krox20 signal can be detected before the formation of the ventral BCC clusters at HH18 (Mauti et al., 2007). The divergence in the onset of expression could be species-specific or due to technical limitations, i.e. different detection thresholds for the chromogenic *in situ* hybridization. Knockout mice or mice carrying a hypomorphic Krox20 allele do not fail to form BCCs but have severe defects in the formation and maintenance of peripheral myelin (reviewed in

Svaren and Meijer, 2008). The specific expression of Krox20 in BCCs is transient, as it gets upregulated by E15.5 in immature Schwann cells of the entire PNS (Topilko et al., 1994).

Erythropoietin

Erythropoietin (EPO, hematopoietic growth factor) is a glycoprotein hormone that is mainly known to control erythropoiesis. In addition, it has also been identified as a candidate neuroprotective compound in certain neurodegenerative diseases. EPO was shown to protect neurons from undergoing apoptosis (Siren et al., 2001). Knabe and coworkers analyzed the expression of EPO and its receptor, EPOR, in a detailed study in mice (Knabe et al., 2005). EPO is expressed during embryogenesis in lateral motor neurons, floor plate, DRG neurons and BCCs. In contrast, EPOR is expressed in precursors of astrocytes, astrocytes, and glial satellite cells. Between E14.5 and E19, all DRG neurons and satellite cells express both, EPO and EPOR. Precursors of astrocytes that mature express EPOR and there is evidence that the astrocytogenesis is controlled by the EPO system (Shibata et al., 1997). It is therefore tempting to speculate that BCCs which are in contact with the precursors of astrocytes induce and control the astrocytogenesis at the transition zone. Indeed, GLAST, a marker that represents cells transforming to astrocytes, showed prominent immunoreactivity at the DREZs and VMEPs (Shibata et al., 1997).

Gdf7

The growth differentiation factor 7 (Gdf7) is a BMP family member that is exclusively expressed in the roof plate of the embryonic spinal cord. It was reported to induce the differentiation of dorsal commissural neurons *in vitro* and the formation of sensory neurons (Lee et al., 1998; Lo et al., 2005). Fate-mapping studies revealed that the progeny of Gdf7-positive NC cells are found in BCCs, peripheral glia, and in nociceptive neurons (Lo et al., 2005). This is a further indication that the NC does not consist of a homologous population of NC cells that acquire their identity once they emigrate from the neural tube. Rather, premigratory NC cells are predefined while they mature in the dorsal neural tube.

Gli3

The zinc-finger-containing transcription factors Gli1, Gli2, and Gli3 are mediators of the Shh/Patched/Smoothened pathway. While Gli1 and Gli2 are activated upon Shh signaling, Gli3 is repressed. The presence of Gli3 is often and indicator for low concentration of Shh. For example, the dorsal neural tube expresses Gli3 due to the antagonizing effect of roof-plate derived BMP on floor-plate derived Shh. Furthermore, BCCs were shown to express Gli3, presumably because of the low concentration of notochord-derived Shh in the ECM (Luo et al., 2006).

Lgi4

Lgi4 encodes a secreted glycosylated leucine-rich repeat protein and is a part of the Schwann cell signaling pathway that controls axon segregation and the formation of myelin. It is mutated in *clp* (claw paw) mice that are characterized by an abnormal limb posture and peripheral hypomyelination. At E14 in mice, Lgi4 is expressed in DRGs, BCCs and in Schwann cell precursors or immature Schwann cells of the peripheral nerves (Bermingham et al., 2006). The function of Lgi4 in BCCs is unknown.

Lingo-1

Lingo-1 is an evolutionary highly conserved protein being exclusively expressed in the nervous system (Okafuji and Tanaka, 2005; Mi et al., 2008). It contains leucine-rich repeats, an immunoglobulin (Ig) domain, a transmembrane domain, and a short cytoplasmic tail. Lingo-1 has been shown to associate with the Nogo-66 receptor (NgR1) and the p75 neurotrophin receptor (p75), and thus to function as an additional component of the NgR1/p75 receptor complex (Mi et al., 2004). Additionally, associations with TrkA and the epidermal growth factor receptor (EGFR) were shown. Interestingly, the expression of Lingo-1 in the DRG is controlled via TrkA (Lee et al., 2007; Mi et al., 2008). Lingo-1 functions as a negative regulator of oligodendrocyte differentiation and myelination, neuronal survival and axonal regeneration (Mi et al., 2008). Despite the fact that Lingo-1 is expressed in BCCs none of the binding partners mentioned above colocalize with it during embryogenesis (Okafuji and Tanaka, 2005), suggesting that it has to associate with other yet unknown molecules.

Monoamine oxidase B

Monoamine oxidases are membrane-bound mitochondrial enzymes that catalyze the oxidative deamination of monoaminergic neurotransmitters. Two types of monoamine oxidases (MAO) exist that have different substrate affinity. While MAOA has a higher affinity for Serotonin and Noradrenalin, MAOB processes β-phenylethylamine and *tele*-methylhistamine. Vitalis and coworkers investigated the distribution of MAOB during mouse embryogenesis (Vitalis et al., 2003). They found MAOB expressed in cells of the hindbrain at the entry zone of the trigeminal nerve into the rhombencephalon that colocalized with Krox20, presumably cranial BCCs. A particularly interesting observation was that no monoaminergic marker colocalized with these BCCs suggesting that the role of MAOB in these BCCs is not the processing of monoaminergic neurotransmitters. However, the authors speculate that MAOB expressed in cranial BCCs could prevent cerebrospinal fluid (CSF)-derived amines from entering the CNS.

The BCCs of the spinal cord express MAOB (Hjerling-Leffler et al., 2005). It is unlikely, that spinal BCCs would exert the same function as suggested for the cranial BCCs since they are not in direct contact with CSF that is present in the central canal of the spinal cord. Furthermore, BCCs seem to retain MAOB expression but downregulate Krox20 once they leave the clusters.

MPZ (Protein zero, P_0)

Protein zero (P_0), an integral transmembrane glycoprotein, belongs to the Immunoglobulin-superfamily of adhesion molecules and is encoded by the myelin protein zero gene (MPZ, CMT1B). It is a major component of the peripheral myelin sheath and is necessary for the formation of compact myelin. Mutations in the MPZ gene are linked to a significant number of peripheral neuropathies resulting in hypomyelination as seen in the Charcot-Marie-Tooth disease (CMT). MBZ was shown to be a direct regulatory target of Krox20 in cooperation with Sox10. Krox20 mutant mice exhibit defects in the myelination of peripheral nerves and have reduced expression levels of MPZ (Topilko et al., 1994). In contrast, the overexpression of Krox20 in Schwann cells induces the expression of MPZ *in vivo* (Parkinson et al., 2003). However, P_0 is already expressed in a subset of migrating NC cells before the onset of Krox20 expression suggesting that additionally other regulatory mechanisms are involved in the control of MPZ (Bhattacharyya et al., 1991). Homophilic binding of P_0 was reported to mediate cell-cell adhesion in both, neuronal and non-neuronal tissues (Filbin et al., 1990; Schneider-Schaulies et al., 1990). Therefore the presence of P_0 in BCCs could possibly mediate adhesion among them.

Pax3 and Pax7

The paired-box-containing transcription factor Pax3 and Pax7 are class-I transcription factors that are repressed by Shh (Briscoe et al., 2000). They are required for the patterning of the neural tube and were shown to be necessary for the induction of NC cells. They are expressed in overlapping areas such as the dorsal neural tube, NC cells, and BCCs. *Splotch* mice lacking Pax3, or Pax7 mutant mice were shown to exhibit defects in the formation of NC cells (Mansouri et al., 1996; Serbedzija and McMahon, 1997). However, their function in BCCs remains elusive.

Semaphorins

The Semaphorins form a large family of guidance molecules that are predominantly known for their role in neuronal repulsion. More than 20 different Semaphorins have been identified and classified into eight subfamilies (Semaphorin Nomenclature Committee, 1999). Classes 1 and 2 are exclusively found in invertebrates, while classes 3, 4, 6, and 7 are expressed in vertebrates. Class 5 Semaphorins are expressed in both invertebrates and vertebrates, whereas class V is a virally encoded Semaphorin. Secreted class-3 Semaphorins are the best described with respect to their function. With a few exceptions they bind to a receptor complex consisting of a Plexin and a Neuropilin. In contrast, transmembrane class-6 Semaphorins bind PlexinAs in a Neuropilin-independent manner (Mauti et al., 2006) and can function as receptors mediating bidirectional signaling (reviewed in Zhou et al., 2008). Semaphorin3B, -3G, and -6A are expressed in BCCs. However, a function in the formation of BCC clusters and the subsequent stabilization of the motor column was discovered only for Semaphorin6A (Bron et al., 2007; Mauti et al., 2007).

Sox8 and Sox10

The HMG-box transcription factors of the SoxE family play eminent roles during the development of the peripheral nervous system. They are involved in the formation of NC cells and interact with other genes to initiate developmental programs involved in the formation of the PNS. Sox8 is upregulated during the formation of the NC and is later found in BCCs and oligodendrocyte precursors (Bell et al., 2000; McKeown et al., 2005). The function of Sox8 in BCCs is unknown.
Sox10 is upregulated in NC cells once they emerge from the dorsal neural tube (Kuhlbrodt et al., 1998) and it was reported to downregulate N-Cadherin which is a crucial step during the EMT of NC cell precursors (Cheung et al., 2005). As soon as NC cells start to differentiate, Sox10 is downregulated. Sox10 was reported to maintain the multipotency of NC cells (Kim et al., 2003). However, BCCs express Sox10, most probably to keep their multipotent state which is necessary for their contribution to DRG neurons and peripheral glial cells.

SSEA-1

Stage specific embryonic antigen 1 (SSEA-1) is a specific cell surface marker for sensory neuroblasts and multipotent cells (Sieber-Blum, 1989). It is expressed in all sensory neuron lineages at all axial levels of quail embryos. Interestingly, the expression pattern differs between quail and rat or chicken embryos, where only a small subpopulation of sensory neurons expresses SSEA-1 (Ernsberger and Rohrer, 1988). NC cells, DRGs, BCCs and their emigrating progeny express SSEA-1 (Oudega et al., 1992).

TrkB

The neurotrophin tyrosine kinase receptor B (TrkB) is a high affinity receptor for BDNF, NT-3, and NT-4. It is expressed in many parts of nervous system such as the NC, NC cells, DRGs, motor neurons, and peripheral glia. The TrkB was reported to be involved in neuronal differentiation and cell survival. Furthermore, the large diameter proprioceptive and mechanoceptive neurons can be distinguished from nociceptors by the expression of TrkB and/or TrkC. However, the function of TrkB in BCCs remains elusive.

Gene	BCCs	NC	NCCs	pGCs	DRGs	MNs	Expression profile	Knockout mice
Cadherin-7	+	-	+	+	(+)	+	(Nakagawa and Takeichi, 1995; Luo et al., 2006)	No
Cxcr4	+	-	+	+	+	+	(Belmadani et al., 2005; Lieberam et al., 2005)	(Zou et al., 1998)[a]
Egr2 (Krox20)	+	(+)[b]	(+)[b]	+[c]	-	-	(Topilko et al., 1994; Maro et al., 2004; Mauti et al., 2007)	(Topilko et al., 1994)
EPO	+	-	-	+[d]	+	+	(Knabe et al., 2005)	(Tsai et al., 2006)
Gdf7	(-)[e]	+	(-)[e]	(-)[e]	(-)[e]	-	(Lee et al., 1998; Lo et al., 2005)	(Lee et al., 1998)
Gli3	+	-	-	-	-	-	(Luo et al., 2006)	No (but see Persson et al., 2002 and references therein)
Lgi4	+	-	-	+	+	-	(Bermingham et al., 2006)	(Bermingham et al., 2006)
Lingo-1	+	-	-	+	+	+	(Okafuji and Tanaka, 2005)	(Inoue et al., 2007)
MAOB	+	-	-	+	+	-	(Vitalis et al., 2003; Hjerling-Leffler et al., 2005)	(Grimsby et al., 1997)
MPZ (P_0)	+	-	+	+	-	-	(Golding and Cohen, 1997; Mauti et al., 2007)	(Giese et al., 1992)
N-Cadherin	+	+	-	+	+	+	(Wanner et al., 2006)	(Luo et al., 2001)[f]
Pax3	+	+	+	+	-	-	(Goulding et al., 1994; Lacosta et al., 2005)	(Serbedzija and McMahon, 1997)
Pax7	+	+	+	-	-	-	(Goulding et al., 1994; Lacosta et al., 2005; Luo et al., 2006)	(Mansouri et al., 1996)
Sema3B	+	-	-	-	-	-	(Bron et al., 2007)	(Falk et al., 2005)
Sema3G	+	-	-	-	+	-	(Bron et al., 2007)	No
Sema6A	+	(+)	+	-	-	(+)[g]	(Mauti et al., 2007)	(Mitchell et al., 2001)
Sox8	+	-	-	-	-	-	(Bell et al., 2000; McKeown et al., 2005)	(Sock et al., 2001)
Sox10	+	-	+	+	+	-	(Kuhlbrodt et al., 1998; Mauti et al., 2007)	(Herbarth et al., 1998; Southard-Smith et al., 1998; Kapur, 1999)
SSEA-1	+	+	+	(+)	+	-	(Sieber-Blum, 1989; Oudega et al., 1992)	(Kudo et al., 2004)[h]
TrkB	+	+	+	+	+	+	(Ernfors et al., 1992; Jungbluth et al., 1997; Straub et al., 2007)	(Klein et al., 1993)

Table 1: Genes expressed in BCCs or their progenitors.

(BCCs) Boundary cap cells, (pGCs) peripheral glia cells, (NC) neural crest, (NCCs) neural crest cells, (DRGs) dorsal root ganglia, (MNs) motor neurons. (a) Embryonic lethal. (b) Radioactive *in situ* hybridization showed a signal (Wilkinson et al., 1989). (c) After E15.5 in all Schwann cells. (d) Starting at E14.5. (e) Gdf7 is exclusively expressed in the NC. Fate-mapping studies revealed that the traced NC cells colonize the DRES (apparently BCCs), dorsal roots, and colocalize with TrkA-positive neurons. (f) The N-Cadherin knockout embryo dies due to severe cardiovascular defects (Radice et al., 1997). Specific expression of N- or E-Cadherin in the heart rescues the early lethality. (g) Transient upregulation at HH25. (h) Disruption of the Fut9 gene results in absence of SSEA-1.

Aim of the thesis

The striking expression pattern of Semaphorin6A in BCCs prompted us to analyze its function during nervous system development.

The aims of the thesis were:

1. Analysis of the expression patterns of Plexins and Neuropilins
2. Functional analysis of Semaphorin6A and PlexinA1

4. Expression Pattern Analysis of Plexins and Neuropilins

Expression patterns of plexins and neuropilins are consistent with cooperative and separate functions during neural development

Olivier Mauti, Rejina Sadhu, Joelle Gemayel, Matthias Gesemann, and Esther T. Stoeckli

Institute of Zoology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

Published in BMC Developmental Biology, 2006, 6:32

BMC Developmental Biology

Research article

Expression patterns of plexins and neuropilins are consistent with cooperative and separate functions during neural development

Olivier Mauti[1], Rejina Sadhu[1], Joelle Gemayel[2], Matthias Gesemann[2] and Esther T Stoeckli*[1]

Address: [1]Institute of Zoology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland and [2]Brain Research Institute, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

Email: Olivier Mauti - Olivier.Mauti@zool.unizh.ch; Rejina Sadhu - rejina.ramachandran@zool.unizh.ch; Joelle Gemayel - gemayel@hifo.unizh.ch; Matthias Gesemann - gesemann@hifo.unizh.ch; Esther T Stoeckli* - Esther.Stoeckli@zool.unizh.ch

* Corresponding author

Published: 17 July 2006

BMC Developmental Biology 2006, **6**:32 doi:10.1186/1471-213X-6-32

Received: 17 April 2006
Accepted: 17 July 2006

This article is available from: http://www.biomedcentral.com/1471-213X/6/32

Abstract

Background: Plexins are a family of transmembrane proteins that were shown to act as receptors for Semaphorins either alone or in a complex together with Neuropilins. Based on structural criteria Plexins were subdivided into 4 classes, A through D. PlexinAs are mainly thought to act as mediators of repulsive signals in cell migration and axon guidance. Their functional role in vertebrates has been studied almost exclusively in the context of Semaphorin signaling, i.e. as co-receptors for class 3 Semaphorins. Much less is known about Plexins of the other three classes. Despite the fact that Plexins are involved in the formation of neuronal circuits, the temporal changes of their expression patterns during development of the nervous system have not been analyzed in detail.

Results: Only seven plexins are found in the chicken genome in contrast to mammals, where nine plexins have been identified. Here, we describe the dynamic expression patterns of all known plexin family members in comparison to the neuropilins in the developing chicken spinal cord.

Conclusion: Our in situ hybridization study revealed that the expression patterns of plexins and neuropilins are only partially overlapping, especially during early and intermediate stages of spinal cord development, supporting both cooperative and separate functions of plexins and neuropilins in neural circuit formation.

Background

The formation of neuronal circuits crucially depends on the correct navigation of axons to their target areas, where they contact individual target cells to establish synaptic contacts. Axonal navigation is based on sequential growth from choice point to choice point. Pathfinding decisions at choice points and along the axonal trajectory are the consequence of molecular interactions between guidance cues presented by the environment and guidance receptors expressed on the growth cones. A multitude of in vitro and in vivo approaches led to the identification of guidance cues that provide directional information for the navigation of growth cones. The Semaphorins are a structurally diverse family of guidance cues. They are subdivided into eight subfamilies, two found in invertebrates, one in viruses, and five in vertebrates. Initially, Sema-

Table 1: Chromosomal localization and EST representation of different plexin genes

plexin	A1	A2	A3	A4	B1	B2	B3	C1	D1
# ESTs	16	23	n.d.	4	4	23	n.d.	6	9
Chick Chr.	12	26	n.d.	1	12	?[1]	n.d.	1	12
Mouse Chr.	6d1	1h6	Xa7.1	6a3.3	9f2	15e3	Xa7.1	10c2	6e3

The number of identified chicken ESTs for each gene and the chromosomal location of the chicken and its corresponding mouse gene are given. Genes that were not detected are abbreviated by 'n.d.' [1]PlexinB2 has not yet been assigned to a chromosome.

phorins were found to be repellents for extending axons. In 1997, Neuropilins were identified as receptors for Semaphorins concurrently in two labs [1-3]; reviewed in [4]. A short time later, a role for Plexins as receptors for Semaphorins was described [5-9]. However, Neuropilins and Plexins had been discovered many years earlier as antigens of monoclonal antibodies raised against proteins from the optic tectum of *Xenopus laevis* [10-12]. In contrast to Neuropilins, which have only been found in vertebrates, Plexins are distributed widely in both vertebrates and invertebrates [13]. The nine Plexins found in vertebrates have been subdivided into four subclasses A-D depending on structural criteria. The largest subfamily is the PlexinA subfamily with four members, followed by the PlexinB subfamily with three members. Subfamilies C and D contain only one member each.

By far the best-studied Plexins are class-A Plexins [14-16]. Their function has been studied predominantly in context of their role as co-receptors (together with Neuropilins) for secreted class-3 Semaphorins [14,16,17]. However, PlexinAs must have functions that are independent of Neuropilins, because they are expressed much more widely in the developing nervous system than Neuropilin-1 and -2. Consistent with this, Plexins were shown to mediate homophilic cell-cell adhesion in a calcium-dependent manner [11]. Furthermore, PlexinAs were shown to mediate effects of membrane-bound class-6 Semaphorins in a Neuropilin-independent manner [18,19].

Until recently, when Sema3E binding to PlexinD1 in a Neuropilin-independent manner was demonstrated during the development of the intersomitic vasculature [20], Neuropilins were thought to be required but not sufficient for class-3 Semaphorin signaling [8,9,21-23]. No signaling component in the cytoplasmic part of Neuropilins could be identified, suggesting that they confer ligand specificity to the complex formed with Plexins, L1, or Off-track [24,25]. In contrast to the secreted class-3 Semaphorins, membrane-associated Semaphorins were shown to bind to Plexins directly [18,19,26,27].

Much less is known about the function of other classes of plexins. An interaction of PlexinB1 with Sema4D has been described, but little is known about the role of PlexinBs in vivo [26,28]. PlexinC1 was demonstrated to interact with Sema7A [7], although the only functional study available to date indicates Integrins rather than PlexinC1 as the function-mediating receptor for Sema7A [29]. PlexinD1 finally was linked to the development of the heart and the vascular system consistent with its predominant expression in endothelial cells [30-32].

As a step toward a better understanding of the diverse roles of Plexins in the developing nervous system, we decided to assess the expression patterns of plexins. Here, we describe the expression of all plexins found in the chicken genome during spinal cord development in comparison with neuropilins.

Results

The avian genome lacks homologues for two mammalian plexins

In order to identify chicken plexins and neuropilins we performed extensive databank searches using the combined information from the BBSRC ChickEST and the genomic database. These searches indicate that the chicken genome encodes a reduced number of plexins compared to mammals, i.e. only seven instead of nine. While homologous chicken genes for the two unique subfamily members plexinC1 and plexinD1 could readily be identified, fewer chicken plexinAs and plexinBs are present in the chicken genome when compared to mammals (Table 1; Figure 1; [33]). The plexinA subfamily contains no matching chicken sequence for plexinA3. Furthermore, no counterpart for plexinB3 could be extracted from chicken databases (Figure 1 and Table 1). Interestingly, the mouse variants of the two missing plexin genes are located on the X chromosome, implying that a homologous region of this chromosome is absent in chicken. Birds use a Z/W sex determination system instead of the X/Y system found in mammals. Moreover, in contrast to mammals, in chicken the female is the heterogametic sex (ZW), whereas the male is homogametic (ZZ), further suggesting that intense chromosomal rearrangements happened during evolutionary development [34]. These changes are also reflected by the fact that the haploid chicken genome contains 38 autosomes compared to only 19 autosomes in mouse [33,35]. Based on the align-

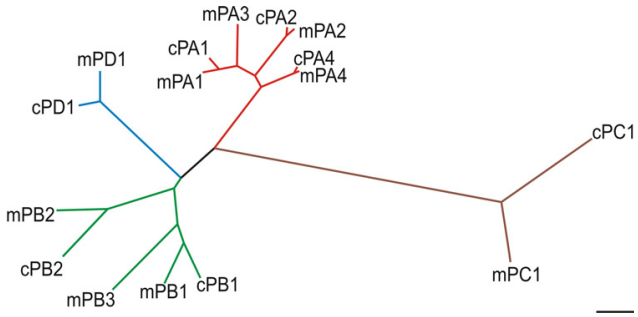


Figure 1
Phylogenetic tree of the plexin superfamily. Plexins were aligned on the basis of their sema domain using the CLUSTAL W program. The scale bar represents a substitution rate of 10 amino acids per 100 amino acid residues. For simplicity only mouse (m) and chicken (c) sequences are depicted but the alignment of rat or human sequences gave similar results (data not shown).

ment of human, mouse, and chicken chromosomes, genes found on the mammalian X chromosomes were assigned either to the chicken chromosome 1 or 4 [33].

The chicken genome with 1.2×10^9 bp is only about 40% as long as the human genome, and with an estimated 20'000 to 23'000 genes the chicken contains fewer genes than the mouse or the human genome, although the difference in gene number is not proportional to the reduction in genome size. The chicken genome contains markedly less repetitive sequence and a reduced number of degraded copies of gene sequence, but also fewer duplicated copies of genes overall [33]. Thus, our finding that two plexins are missing is most likely due to their actual absence in the genome rather than our inability to detect them in the databases. Moreover, all attempts to clone the missing chicken homologues using degenerate primers for RT-PCR failed, again implying that the missing genes are indeed not represented in the chicken genome.

Interestingly, while conservation between different chicken plexinAs was in the range of 70 to 90%, depending on the plexin parts used for alignment, conservation between chicken plexinBs was only around 50% (Table 2). This value is not much higher than the values obtained when plexinBs were compared to plexins in other subclasses, suggesting that although placed into the same subclass, plexinB1 and B2 might actually be members of different subclasses.

The expression patterns of plexins are dynamically regulated during early spinal cord development

We started to analyze plexin expression in the lumbosacral spinal cord at stage 18 [36]. At this time, motor neurons are born in the ventral spinal cord and start to extend axons. The first plexins detected in motor neurons were plexinA1 and A2 (Figure 2, compare [Additional file 1] for sense controls). In addition, motor neurons expressed neuropilin-1 (npn-1), but not npn-2. When compared with markers for precursors and mature neurons (Figure 3), respectively, plexinA1 and A2 were clearly expressed already in precursors of motor neurons, whereas npn-1 was restricted to more lateral, mature motor neurons.

More dorsally, plexinA1, A2, and B1 were expressed in Pax6-positive precursor cells (compare to Figure 3A). In addition, cells in the dorsal spinal cord expressed plexinA1, A2, B1, as well as npn-2 (Figure 2). These cells expressed Pax7 (Figure 3D) but did not express neurofilament proteins (Figure 3E) and therefore represent precursors of dorsal interneurons [37]. At the lumbosacral level of the spinal cord, dorsal commissural neurons derived from these Pax7-positive precursors and characterized by the expression of the cell adhesion molecule Axonin-1 start to extend axons at stage 19 (Figure 3F; [38]). These axons grow toward the floor plate in response to chemoattractants derived from the floor plate, Netrin-1 (reviewed in [39]) and Sonic hedgehog [40]. The majority of these axons have reached the floor plate by stage 22. At this stage, dorsal commissural neurons identified by axonin-1 expression [38,41] expressed all three members of the plexinA class but neither npn-1 nor npn-2 (Figure 4; compare [Additional file 2] for sense controls). Interestingly, plexinA1 and A2 were now also expressed in the floor plate, although plexinA1 was found only in lateral but not medial floor-plate cells (Figure 5). Furthermore, the floor plate was also the earliest site of plexinB1 and C1 expression in the spinal cord (Figure 4 and 5). In contrast to all other plexins, plexinB1 was expressed strongly in the entire ventricular zone and at low levels in almost all cells of the spinal cord at stage 22 and later.

Motor neurons innervating the hindlimb express a large variety of plexins and neuropilins

After stage 22, motor axons reach the plexus region where they have to sort out according to their target muscles. While the first decision is primarily a choice to grow either dorsally or ventrally, more refined pathways are chosen at stages 25/26, when individual nerves begin to form [42,43]. At that time, motor neurons can be separated into different subpopulations based on the expression pattern of transcription factors [44-46] or type-II cadherins [47]. At stage 25, all plexins and both neuropilins were expressed in motor neurons (Figure 6). PlexinA1, A4, and, at low levels, B2 were expressed in a pattern overlapping with Isl1-positive motor neurons (Panel C in Figure 6) that coincided with the expression of SC1 a surface marker for motor neurons C1 (not shown). In contrast, the expression of plexinD1 and npn-1 was strong in medial

Table 2: Conservation of different domains between Plexin superfamily members

			SEMA domain			
plexinA2	57/71					
plexinA4	56/70	59/76				
plexinB1	28/47	28/48	27/47			
plexinB2	28/47	30/49	30/48	36/55		
plexinC1	13/23	12/22	13/22	12/20	12/21	
plexinD1	24/38	23/40	23/40	27/43	24/40	15/28
	plexinA1	plexinA2	plexinA4	plexinB1	plexinB2	plexinC1
			PSI domain			
plexinA2	64/73					
plexinA4	74/87	62/77				
plexinB1	50/61	44/55	50/58			
plexinB2	44/65	39/56	39/60	48/66		
plexinC1	42/70	42/76	46/65	48/65	38/57	
plexinD1	37/53	41/61	37/55	40/51	39/53	39/56
	plexinA1	plexinA2	plexinA4	plexinB1	plexinB2	plexinC1
			SP domain			
plexinA2	88/95					
plexinA4	82/94	86/95				
plexinB1	56/72	56/73	53/73			
plexinB2	56/74	56/75	56/76	66/79		
plexinC1	49/69	48/68	49/70	45/65	48/66	
plexinD1	56/75	56/74	58/75	55/74	55/74	57/77
	plexinA1	plexinA2	plexinA4	plexinB1	plexinB2	plexinC1

Pairwise alignment of individual domains of chicken Plexin proteins indicate that the intracellular SP (sex-plexin) domain is highly conserved between the different family members, whereas the two extracellular domains, SEMA and PSI (plexin/semaphorin/integrin), show far less conservation. Interestingly the conservation between individual domains of chicken plexinB1 and B2 is only slightly higher than the homology between class A and class B plexins, suggesting that chicken plexinBs are evolutionary quite distinct. Numbers indicate percentage of identical/ conserved amino acids.

motor neurons labeled by MNR2 but not by Isl1 (Panel D in Figure 6). Npn-2 appeared to be expressed predominantly in a dorsolateral subset of Isl1-positive cells. PlexinA2 and C1 were scattered throughout the ventral horn without clear resemblance to either the Isl1 or the MNR2 pattern and expression levels were rather low. This was also true for plexinB1 that was expressed throughout the neural tube with higher levels found in dorsal commissural neurons, the floor plate, and the ventricular zone. In addition to plexinB1, the floor plate maintained expression of plexinA2, and C1 at stage 25. PlexinA1 was still detectable in lateral floor-plate cells, although at lower levels. Dorsolateral commissural neurons still expressed all plexinAs, plexinB1, and C1, but no neuropilin.

During intermediate stages of spinal cord development plexin expression is reduced

The early phase of spinal cord development that we analyzed (stages 18 – 25) is characterized by the birth and differentiation of neuronal populations [48-50,37]. This two-day period (E3-E5) revealed fundamental changes in the expression patterns of plexins and neuropilins (compare Figures 2, 4, and 6). In contrast, the expression patterns changed much less during the next two to three days, the intermediate phase of spinal cord development. At stage 30 (Figure 7), the expression pattern of plexinAs remained the same as at stage 25, except for the decrease of plexinA1 in the dorsal half of the spinal cord. PlexinB1 persisted only in the ventral ventricular zone, and plexinB2 disappeared altogether. PlexinC1 was now expressed diffusely throughout the gray matter with higher levels in the lateral motor column and the floor plate. After its transient expression in motor neurons at stages 25 (Figure 6) and 26 (not shown) plexinD1 was no longer expressed in the spinal cord but was restricted to endothelial cells of the blood vessels as described for the mouse [30]. The expression of npn-1 and npn-2 at stage 30 (Figure 7) remained largely complementary in different populations of motor neurons as already indicated at stage 25 (Figure 6). The expression patterns of the neuropilins in motor neurons did not resemble those of any of the plexins. A more detailed analysis of the individual subpopulations expressing plexins and neuropilins would exceed the scope of this study and would require double and triple staining for transcription factors of the ETS and LIM homeodomain families [45,51,52] or comparison with the analysis of type-II cadherin expression patterns in motor neuron pools [47]. The analysis of whole-mount preparations of stage 26 spinal cords did

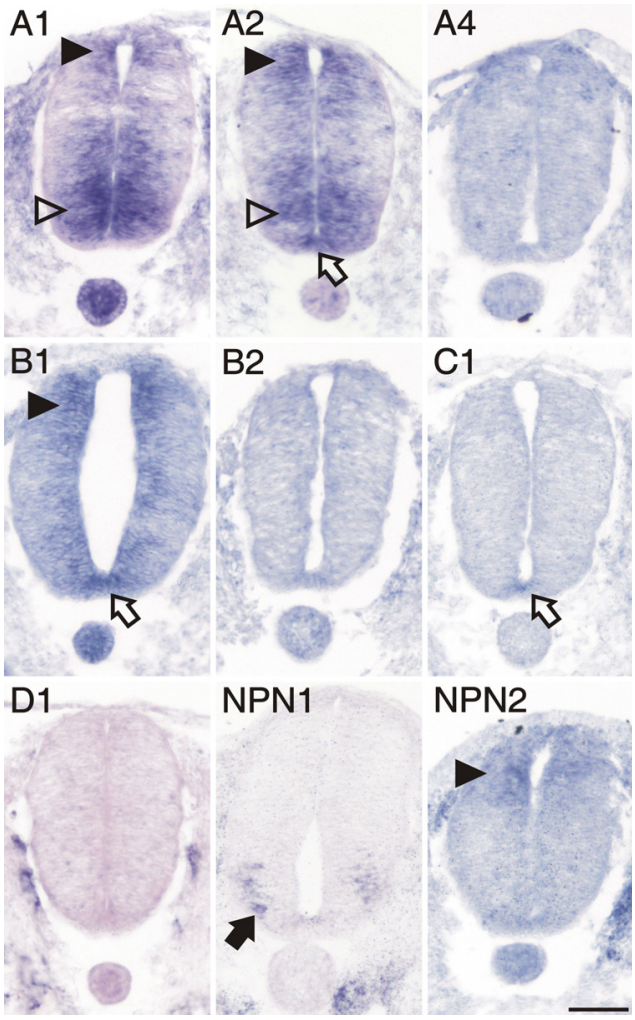


Figure 2
Expression of plexins and neuropilins in the spinal cord at stage 18 analyzed by in situ hybridization. PlexinA1, A2, and B1 were detectable with very similar patterns in the dorsal spinal cord at stage 18, the earliest stage we investigated (arrowheads). PlexinA1 and A2 were also expressed in early motor neurons of the ventral spinal cord (open arrowheads). PlexinA2, B1 and C1 were the only plexins expressed in the floor plate (open arrow). In contrast to plexinA1 and A2, npn-1 (NP1) was expressed only in motor neurons that had migrated laterally (arrow). Npn-2 (NP2) was expressed at low levels in the dorsal spinal cord similar to plexinA1, A2, and B1 (arrowhead). Adjacent sections hybridized with the respective sense probes are shown in [Additional file 1]. Bar 50 μm.

not reveal any substantial changes in the expression patterns of plexins and neuropilins throughout the lumbosacral level [see Additional file 3].

Late stages of spinal cord development

During late stages of spinal cord development, between stages 35 (Figure 8) and 40 (Figure 9), motor axons have reached their target muscles and sensory afferents terminate in their specific target layers of the gray matter. Expression patterns of plexins and neuropilins still changed. Overall their levels decreased and their spatial extent became more restricted. Fundamental changes in expression were observed for npn-1 that was found in the dorsal spinal cord during late stages of spinal cord development, but not during early and intermediate stages, where it had been restricted to the ventral spinal cord.

Another significant change was found for plexinB1 that was detected in cells located in the funiculi all around the spinal cord at stage 40 (Figure 9). The expression of plexinB1 (Figure 9A for higher magnification) correlated with areas of O4 expression, a marker for oligodendrocytes (Figure 9B) but not with GFAP expression, a marker for astrocytes (Figure 9C).

At late stages of development, the expression of plexins was restricted to the dorsal gray matter, or was turned off altogether (plexinA4, B2). Strong expression only persisted for plexinA2 and C1 in the dorsal horn, in laminae I – III (Figure 9; [53]).

DRGs exhibit a dynamic plexin expression pattern

Dorsal root ganglia expressed a variety of plexins during different stages of development. During early stages (stages 22–25), when sensory neurons extend processes into the periphery but before collaterals of central processes are formed ([54], and references therein), plexinA1, A4, and npn-2 transcripts were found at high levels; low levels were detectable for plexinA2, B1, and npn-1 at stage 22 (Figure 4). At stage 25, expression patterns were unchanged for plexins but levels had switched for neuropilins, i.e. npn-1 was expressed at higher, npn-2 at lower levels at stage 25 compared to stage 22 (Figure 6, compare to Figure 4).

At stage 30, when sensory neurons start to extend collaterals into the gray matter of the spinal cord (see [54] and references therein), high levels of plexinA4 were maintained in DRGs (Figure 7), whereas levels of plexinA1 and B1 had decreased. PlexinC1 expression levels started to increase (compare Figures 6, 7, and 8). PlexinA2 remained restricted to a subset of cells. Interestingly, the expression of npn-2 became restricted to sensory neurons located in the dorsal-most part of the DRG at stage 30. As nociceptive, mechanoreceptive, and proprioceptive neurons are segregated in the chicken DRG, these cells are most likely nociceptive neurons [55,53].

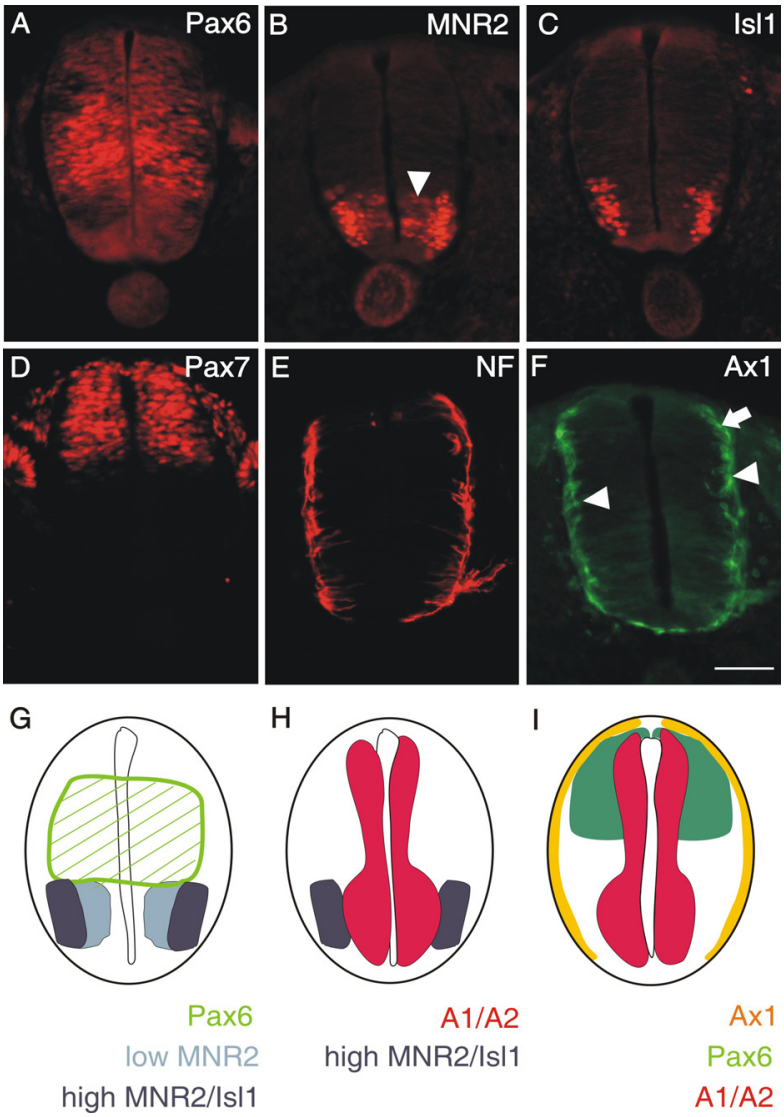


Figure 3

PlexinA1 and A2 are already expressed in neural precursors. At stage 18, the homeodomain protein Pax6 labels precursor cells in the intermediate zone of the developing spinal cord (A; [60]). Its ventral expression boundary that is defined by the morphogen Shh released from the floor plate reaches the area with the progenitors of motor neurons expressing MNR2 (B and G). Low levels of MNR2 proteins are seen in progenitor cells located medially (arrowhead in B), where motor neurons are born. MNR2 protein persists and accumulates in postmitotic motor neurons expressing Isl1 (C). In the dorsal spinal cord, Pax7 expression marks a population of precursor cells that give rise to dorsal interneuron subpopulations (D; [37]). Note the decline of Pax7 staining toward the periphery of the spinal cord. Even at stage 19, postmitotic neurons expressing neurofilament proteins are found only at the peripheral margin of the spinal cord (E). At this stage, the dorsolateral population of commissural axons, characterized by the expression of Axonin-1 (arrow in F), starts to extend axons toward the floor plate [38]. At the same, more ventrally located interneurons expressing Axonin-1 also extend axons but their pathway has not been characterized in detail (arrowheads). A comparison of the expression of Pax6 (green hatched area), MNR2 (light and dark blue, characterizing low and high protein levels, respectively) and Isl1 (dark blue) is shown in G. For clarity the mRNA expression domains of plexinA1 and A2 have been added in H (red). Significant overlap was found between the plexin expression domain and early motor neurons characterized by MNR2 staining (H). In the dorsal spinal cord domains of Pax7 (green) and Axonin-1 protein expression (yellow) are compared to the domains expressing plexinA1 and A2 mRNA in I. Plexins do not extend to the periphery of the neural tube where postmitotic neurons expressing neurofilament or axonin-1 are found. Bar in A through F 50 μm.

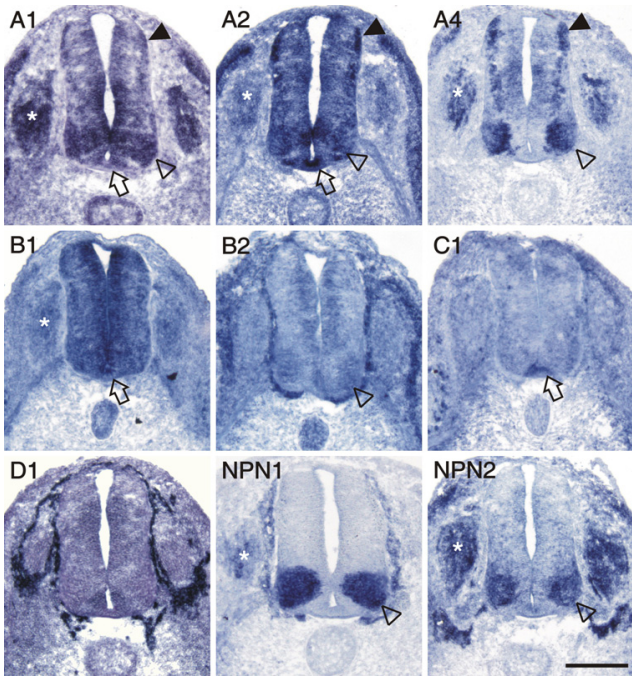


Figure 4
Both neuropilins and all plexins, except D1, were expressed in the spinal cord at stage 22. Motor neurons expressed plexinA1, A2, A4, B2, and both neuropilins (open arrowheads). Levels of plexinB2 were very low and plexinA2 appeared to be expressed only in a subset of motor neurons. All class-A plexin were found in dorsal commissural neurons (arrowheads). PlexinA1 and A2, but not A4, were detected in the floor plate (open arrows). Furthermore, the floor plate expressed plexinB1 and C1. PlexinB1 was found throughout the spinal cord with higher levels in the ventricular zone. PlexinD1 was not expressed in the spinal cord at all, but was restricted to endothelial cells and cells ensheathing the spinal cord. The DRGs expressed plexinA1, A2, A4, B1, npn-1 and npn-2 (asterisks). For a comparison sections hybridized with the respective sense probes are shown in [Additional file 2]. Bar 200 μm.

At stage 35, plexinA1 and A2 were only found in a few cells located in the periphery of the DRG, where proprioceptive neurons are located (Figure 8; [47,53,55]).

Discussion

PlexinAs have a more widespread distribution compared to neuropilins

The distribution of class-A plexins has been studied in more detail than other plexin subfamilies [15,56,57]. Their function has been analyzed predominantly in context of their role as co-receptors (together with Neuropilins) for secreted class-3 Semaphorins [9,56,58,59] but see [18,19,27]. However, based on their much more dynamic and more widespread expression, a Neuropilin-independent role of PlexinAs is evident. In particular, the observation that plexins are expressed in the floor plate is rather unexpected, as the floor plate is the intermediate target of commissural axons, and therefore, the site where ligands for axonal guidance receptors should be expressed. A receptor function of Plexins on floor-plate cells at stage 22 is not obvious, as floor-plate cells do not migrate or undergo structural remodeling at this time. Floor-plate development is terminated much earlier [41,49,50,60,61].

As mentioned above, the chicken genome contains only three members of the A class: plexinA1, A2, and A4. The available expression patterns of class-A plexins [15,56,58,62] fail to reflect the dynamics of plexinA-expression changes shown in this study. At E12.5, plexinA1 is reported to be barely detectable in the mouse spinal cord and DRGs [56,58]. PlexinA2 was found in the roof plate and in interneurons of the ventral spinal cord but not in motor neurons of the trunk region [58]. At more rostral levels, plexinA2 was found in the dorsal spinal cord and in DRGs. Reports of plexinA3 expression were contradictory. Brown et al. [58] reported plexinA3 expression in the ventricular zone and in the floor plate, whereas Cheng et al. [56] reported plexinA3 to be expressed throughout the spinal cord (except for the ventricular zone). The reason for this discrepancy is unclear. Compared to the expression pattern in chick, the pattern of plexinA3 reported by Brown et al. [58] is almost identical to the one we found for plexinB1 at stage 25 (Figure 6). The pattern of plexinA4 in the E12.5 spinal cord of the mouse was restricted to a single population of cells in the lateral spinal cord and to the DRGs [56,62]. With respect to the development of sensory afferents to the spinal cord, E12.5 in the mouse is comparable to stage 30 in the chick. The development of motor neurons at E12.5 is closer to stage 25. We therefore used both stage 25 and stage 30 spinal cords to compare the plexinA patterns between mouse and chick. While plexinA2 and A4 patterns did not change considerably between stages 25 and 30, plexinA1 levels decreased strongly in the dorsal spinal cord and in the DRGs. Consistent with the pattern reported by Brown et al. [58] in mouse, plexinA2 was expressed in the roof plate at stage 25. However, in contrast to the chick, mouse plexinA2 was not expressed in the floor plate.

PlexinBs are only transiently expressed in neurons

The expression of class-B plexins is difficult to link to any specific function. They are expressed in neurons, although only transiently, and in glial cells. The most prominent and longest lasting expression for a class-B plexin is seen in the ventricular zone, where plexinB1 is expressed from stage 22 to stage 35. This pattern is consistent with the mouse, where plexinB1 was found in the ventricular zone of the spinal cord at E13.5 [63]. At stage 40 in the chick, plexinB1 is restricted to cells in the white matter, presum-

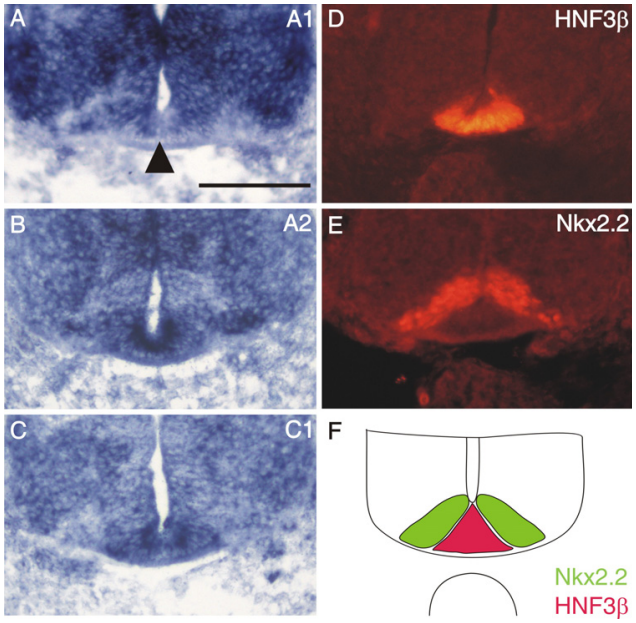


Figure 5
The expression of plexinA1 in the floor plate is not uniform. At stage 22, plexinA1 (A) is expressed strongly in lateral floor-plate cells but present only at very much reduced levels or absent altogether in medial floor-plate cells (arrowhead). In contrast to plexinA1, plexinA2 (B) and C1 (C) are found throughout the floor plate at high levels. The floor plate is identified by the expression of the transcription factor HNF3β (D, red in F; [41]). The area exhibiting reduced levels of plexinA1, A2, and C1 adjacent to the floor plate represents area p3, characterized by the expression of the transcription factor Nkx2.2 (E, green in F; [41,50]). Bar 100 μm.

ably oligodendrocytes. This is in contrast to the expression of plexinB1 in mouse [63]. In mouse, plexinB3 is expressed in cells of the white matter resembling the expression of plexinB1 in chick. Furthermore, the expression of plexinB2, which has been reported to match the expression of plexinB1 in the ventricular zone of E13.5 mice [63], differs in chick, where levels of plexinB2 are generally low in the spinal cord and peripheral ganglia. A very weak transient expression of plexinB1 and B2 is detectable throughout the gray matter and in motor neurons between stages 22 and 25, respectively. During that time expression is also found in the DRGs and along the ventral roots, which would indicate that these cells are early Schwann cells aligning with motor axons. In contrast to observations in the mouse [63] plexinBs are not expressed during the time when sensory afferents target their specific layers in gray matter, as no expression is detectable between stages 30 to 35. In the chick, collaterals of primary sensory axons do not form before stage 29 [54]. Thus, a contribution of both plexinB1 and B2 to the formation of central sensory connections seems rather unlikely in the chick in contrast to mouse, where plexinB1 was found in DRGs from E13.5 to E15.5 [63].

PlexinC1 is not expressed in early stages of neural development

In contrast to plexinAs, which are expressed during the time when neurons extend their axons, plexinC1 is expressed only during late stages of neural development. Neither commissural neurons nor motor neurons express plexinC1 during the time when they approach their first intermediate target, the floor plate and the plexus region, respectively. Interestingly, strong expression of plexinC1 is seen in the floor plate at stage 22 (Figures 4 and 5C), i.e. when the majority of the axons from dorsolateral commissural neurons are in the floor plate [38]. The expression in the floor plate persists through stage 25, when commissural axons have crossed the midline and turned into the longitudinal axis [41]. A massive increase in plexinC1 expression in DRGs is found at stage 35, a time when all other plexins are downregulated compared to their earlier expression levels in DRGs. PlexinC1 expression in DRGs persists at stage 40. Thus, plexinC1 starts to be expressed in all neuronal subpopulations that we analyzed when axons have completed the navigation to their targets suggesting that plexinC1 might be involved in target recognition rather than pathfinding. This would be consistent with the finding of Pasterkamp et al. [29] who reported that the effect of Semaphorin7A, which binds to PlexinC1 with high affinity [7], on axon growth was independent of PlexinC1 but rather mediated by Semaphorin7A's interaction with Integrins.

PlexinD1 is expressed predominantly in endothelial cells

PlexinD1 is expressed transiently in motor neurons in a subpopulation-specific manner between stages 24 and 26. It is not expressed in the spinal cord during initial axon growth of motor neurons. Only after motor axons have reached the plexus region, some medially located cell populations express plexinD1 at stage 25. As described in mouse [30,31], plexinD1 is predominantly expressed in endothelial cells of the developing intersomitic blood vessels during early stages of development and in blood vessels in general during later stages.

The expression of neuropilin-2 in chick is much more restricted than in mouse

The expression of npn-2, but not npn-1, in the developing chicken spinal cord differs considerably from the expression pattern reported in mouse. Neuropilin-1 is expressed in DRGs and motor neurons in E10.5 and E12.5 mouse spinal cord [1,58] in a pattern that is similar to the expression in chick at stages 22–25 (Figures 4 and 6). The expression seen in mouse spinal cord at E13.5 [1] is virtually the same as seen in chick at stage 35 (Figure 8).

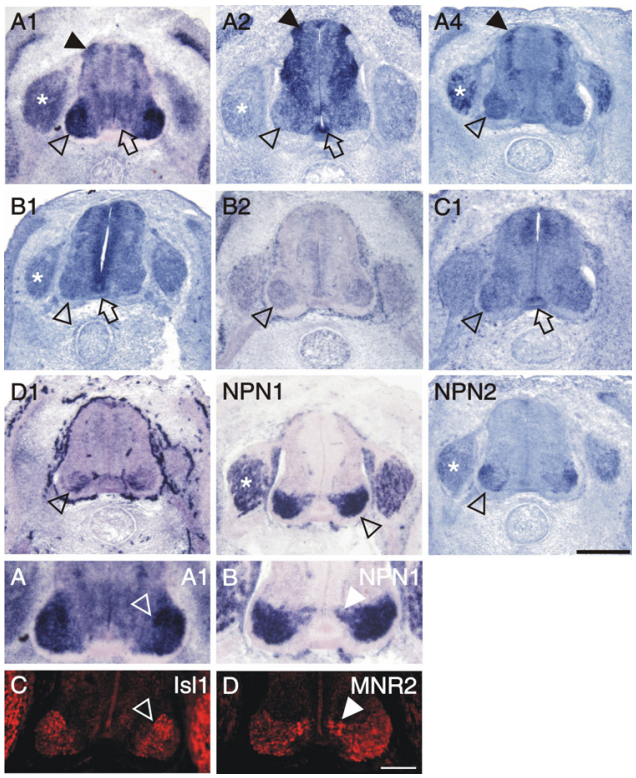


Figure 6
Stage 25 was the only stage when all plexins and neuropilins were expressed in the spinal cord. The most prominent mRNA levels were found for plexinAs. They were all detectable in dorsolateral commissural interneurons (arrowheads) and in motor neurons (open arrowheads). Expression of plexinA2 was restricted to some scattered cells in the ventral horn, but absent from lateral areas. Only subpopulations of motor neurons expressed plexinD1, npn-1, and npn-2. PlexinB1, B2, and C1 were found in all motor neurons although at low levels (open arrowheads). Panels A and B are higher magnifications of the sections hybridized with plexinA1 and npn-1, respectively, in comparison to Isl1 (C) and MNR2 (D) staining in adjacent sections. The expression domains of plexinA1, A4, and npn-2 are more similar to the domains expressing Isl1, whereas those for plexinA2, D1, and npn-1 overlap with the more medial motor neurons expressing MNR2. Floor-plate expression was still found for plexinA1 (lateral floor-plate cells only), A2, B1, and C1 (open arrows). The presence of plexinA1, A2, A4, B1, npn-1, and npn-2 in DRGs is indicated by asterisks. Bar 200 μm.

In contrast to the widespread expression of npn-2 in the mouse spinal cord between E10.5 and 12.5 [1,58,64,65], the expression in the embryonic chicken spinal cord is much more restricted at comparable stages. In mouse, npn-2 is expressed very strongly in dorsal commissural neurons, in ventral populations of interneurons, in motor neurons, and in the floor plate. Functional studies have identified a role for npn-2 in commissural axon pathfinding in the mouse [66]. In chick, npn-2 is not expressed in the dorsal spinal cord at comparable stages. It is restricted to some pools of motor neurons and becomes further restricted with increasing embryonic age. Moreover, npn-2 is never expressed in the embryonic chicken floor plate.

Conclusion

Our analysis of plexin and neuropilin expression during development of the spinal cord reflects their dynamic regulation that coincides with time windows of axon growth and guidance of different neuronal populations. PlexinAs are expressed more widely than any other class of plexins both temporally and spatially. Notably they are also expressed more widely than the neuropilins, indicating that they must have functions that are Neuropilin-independent. This aspect is underestimated when global expression patterns are compared rather than the detailed subpopulation-specific expression patterns. PlexinBs are expressed during early and intermediate stages of spinal cord development but always at low levels and rather ubiquitously. PlexinC1 is expressed predominantly during late stages of development, whereas plexinD1 is expressed only very transiently in the spinal cord.

Methods

Assembly of chicken plexin cDNAs

cDNA sequences for chicken plexins were assembled using the combined information from the ChickEST [67] and the chicken genomic database [68]. Seven different genomic regions coding for plexins were identified using the tblastx alignment algorithm on available vertebrate plexins. The corresponding genomic fragments were downloaded and analyzed using the Genscan gene prediction program [69]. Putative cDNA and protein sequences were compared to the corresponding mammalian homologues and Genscan prediction errors were corrected by manual inspection of the intron/exon boundaries. Gaps in the assembled sequences, due to inaccurate or incomplete genome sequencing, were filled by corresponding EST sequences where possible. A total of 85 chicken ESTs covering parts of 7 different plexins were identified. Among these, 65 contained parts of the coding sequence, whereas the rest covered only parts of the UTR. Sequence alignment of genomic and EST sequences was done using the SeqMan software (Lasergene, DNASTAR). Based on overlapping EST sequences that were supplemented with genomic sequences, 3'UTR sequences were added to the coding sequence. UTR sequences were terminated at the first polyadenylation AATAAA/ATTAAA sequence that followed after verified chicken UTR EST sequences. Using this combined approach a total of 5 complete and 2 partial cDNA sequences for plexins could be assembled.

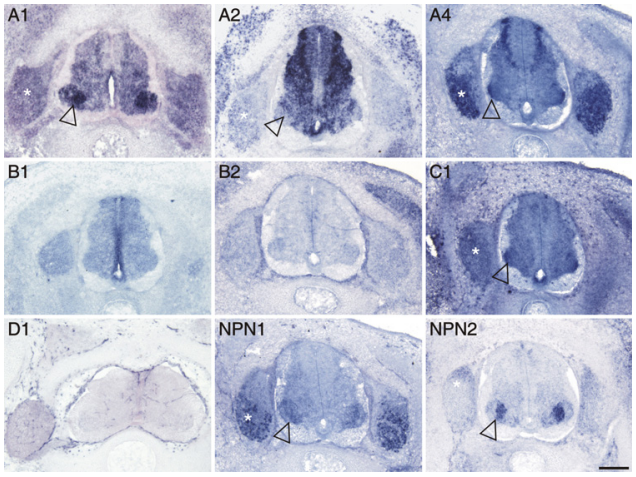


Figure 7
Expression of plexins and neuropilins in the spinal cord at stage 30. The transition from early to late phases of spinal cord development was characterized by restriction of plexin-expressing areas. PlexinB2 and D1 were no longer expressed in the spinal cord, the expression levels of plexinB1 had declined considerably compared to stage 25. PlexinA1 was strongly reduced in the dorsal spinal cord, but remained expressed at high levels in motor neurons (open arrowhead). Motor neurons still expressed plexinA2, A4, C1, npn-1 and npn-2 in a subpopulation-specific manner. In DRGs plexinA1, A2, A4, C1, npn-1, and npn-2 were expressed (asterisk). Bar 200 μm.

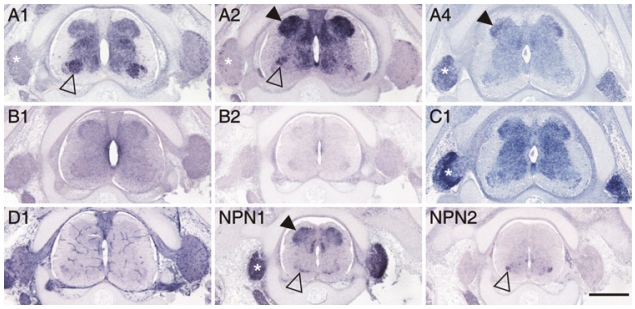


Figure 8
Expression of plexins and neuropilins in the spinal cord at stage 35. Plexins became more restricted during late stages of spinal cord development. Motor neurons expressed plexinA1, A2, npn-1, and npn-2 in a subpopulation-specific manner (open arrowhead). PlexinA2 expression levels were very high in the dorsal horn (arrowhead), whereas plexinA4 was found only in a restricted area of the lateral dorsal horn. PlexinC1 was still found throughout the gray matter. In contrast to earlier stages, npn-1 was now expressed in the dorsal spinal cord (arrowhead). Expression of plexinA1, A2, A4, C1, and npn-1 in DRGs is indicated by asterisks. Bar 500 μm.

Phylogenetic tree assembly and analysis of domain identity

The domain structure of representative members of the chicken and mouse plexin superfamily was obtained using the SMART program [70]. Individual domains were extracted from the sequence using predicted domain boundaries. Conserved domains from the different plexin subfamilies were aligned using the CLUSTAL W alignment algorithm [71,72] provided by the MegAlign software (Lasergene, DNASTAR). Obvious mistakes in domain boundary prediction were manually adjusted. For better representation alignment files were exported into the TREEVIEW software, enabling the graphical representation of the unrooted tree [73]. Identical and conserved amino acids within individual domains were determined by pairwise alignment using the bl2seq program [74].

Preparation of in situ probes

The chicken cDNA plasmids derived from the ESTs ChEST53D13 (plexinA1), ChEST128L21 (plexinA2), ChEST1014M19 (plexinA4), ChEST890P9 (plexinB1), ChEST799I19 (plexinB2), ChEST860K1 (plexinC1), ChEST867E24 (plexinD1), ChEST110K21 (npn-1), and ChEST675H12 (npn-2) were linearized using restriction endonucleases NotI or EcoRI (Roche). The linearized plasmids were used as templates to produce DIG-labeled in situ probes. For this purpose 1 μg of each cDNA was mixed with 3 μl DIG RNA labeling mix (Roche; 10 mM NTP). Three μl T3 or T7 RNA polymerase (Promega, 20 U/μl), 6 μl of 5× transcription buffer (Roche), 0.8 μl RNasin (Promega, 40 U/μl), 3 μl 100 mM DTT and DEPC-treated H_2O were added to a final volume of 30 μl. After incubation at 37°C for 2 hours, 3 μl RNase-free DNaseI (Promega, 1 U/μl) were added to the mix, and incubated at 37°C for 30 minutes. Nuclease treatment was stopped by the addition of 2 μl 0.5 M EDTA (pH 8.0). The cRNA probes were LiCl-precipitated and dissolved in 50 μl of DEPC-treated H_2O.

In situ hybridization

Chicken embryos were collected at indicated embryonic stages, fixed for 2 hours in 4% paraformaldehyde (PFA) and cryoprotected with 25 % sucrose in 0.1 M sodium phosphate buffer overnight. The embryos were embedded in Tissue-Tek O.C.T compound (Sakura), cut transversally into 20–25 μm sections at a temperature of -20°C, and mounted on Superfrost Plus microscope slides (Menzel-Glaeser). After drying the slides for 30 minutes at 37°C, they were stored at -20°C until further use. For all steps until hybridization we used diethyl pyrocarbonate (DEPC) treated H_2O and stock solutions. Sections were washed in PBS for 5 minutes and postfixed in 4% PFA for 30 minutes. They were washed twice in PBS and once in H_2O for 5 minutes each. Sections were then acetylated for 10 minutes in 1% triethanolamine containing 0.25% (vol/vol) acetic anhydride. After two washes for 5 minutes

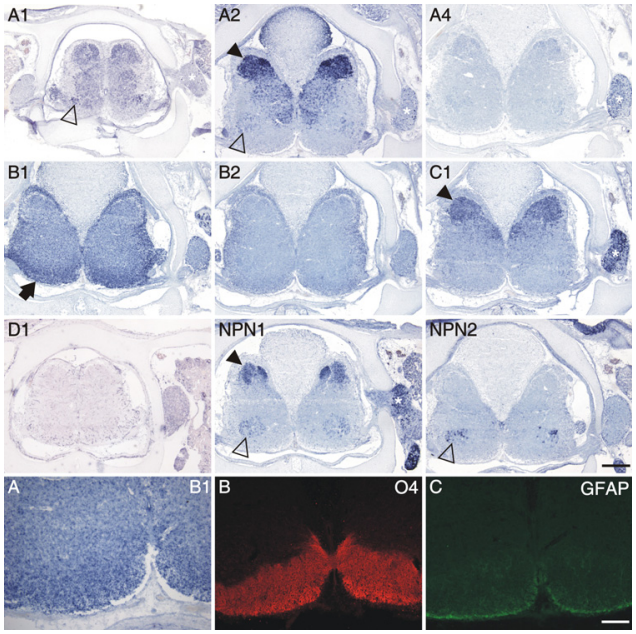


Figure 9
Expression of plexins and neuropilins in late stages of spinal cord development. At stage 40, the latest stage we analyzed, plexin expression still changed. The most prominent change was found for plexinB1, which was now restricted to the white matter (arrow; high magnification shown in A). Strong expression in the dorsal spinal cord was still found for plexinA2, C1, and npn-1 (arrowhead). Few positive motor neurons were still found for plexinA1, A2, npn-1, and npn-2 (open arrowheads). DRGs maintained expression of plexinA4, C1, and npn-1 (asterisk). Single scattered cells were still expressing plexinA1 and A2. Panels A – C show high magnifications of a section processed by in situ hybridization to detect plexinB1 (A). Adjacent sections were stained with antibodies recognizing the oligodendrocyte marker O4 (B) and the astrocyte marker GFAP (C). Bar 500 μm for in situ hybridizations of plexins and neuropilins and 200 μm for panels A-C.

each in PBS and a 5-minute wash in 2× SSC (0.3 M NaCl, 0.03 M tri-sodium citrate, pH 7.0), sections were prehybridized for 3 hours with 300 μl prehybridization solution per slide. To avoid evaporation of the solution, slides were covered with parafilm (pretreated with 7% H_2O_2 in H_2O) for prehybridization and subsequent hybridization. Hybridization was for at least 16 hours. The prehybridization solution comprised of 5× Denhardt's, 250 μg/ml yeast tRNA, 500 μg/ml herring sperm DNA, 5× SSC, and 50% formamide. For hybridization, 200–500 ng/ml of each cRNA probe was added to the prehybridization solution, 300 μl placed on each slide and covered with parafilm. Slides were hybridized at 56°C in a humidified chamber containing 50% formamide/5× SSC. After hybridization, sections were washed for 5 minutes in 5× SSC at 56°C, 5 minutes in 2× SSC at 56°C, 5 minutes in 0.2× SSC at 56°C, 20 minutes in 50% formamide/0.2× SSC at 56°C, 5 minutes in 0.2× SSC at room temperature, and finally washed two times in detection buffer (0.1 M Tris-base, 0.15 M NaCl, pH 7.5) for 5 minutes each at room temperature. Sections were then blocked for 1 hour in blocking buffer (3% milkpowder in detection buffer) and incubated for 1 hour in anti-DIG-AP antibodies (Roche) diluted 1:5000 in blocking buffer (300 μl/slide) in a humidified chamber. After two 15-minutes washes in detection buffer, sections were washed for 5 minutes in alkaline phosphatase (AP) buffer (0.1 M Tris-base, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5). For the color reaction, sections were incubated 12–24 hours in a dark humidified chamber in AP buffer (500 μl/slide) containing 337.5 μg/ml nitroblue tetrazolium (NBT; Roche), 175 μg/ml 5-bromo-4-chloro-3-indoyl phosphate (BCIP; Roche), and 240 μg/ml levamisole (Sigma). The reaction was stopped by washing sections 2 times for 10 minutes each in TE buffer (10 mM Tris-base, 1 mM EDTA, pH 8.0), followed by a short dip in H_2O. Finally, the sections were coverslipped with an aqueous mounting medium (Celvol). Reaction times were the same for all stages for a given probe, except for plexinA1 and A2. For these two probes reaction times for stage 18 were longer than those for older stages to optimize signal to noise rations. Thus, expression levels at st18 cannot be directly compared to older stages.

Immunohistochemistry

Antibodies recognizing Pax6, Pax7, MNR2, Isl1, and SC1/Ben were obtained from the Developmental Studies Hybridoma Bank. The monoclonal antibody RMO270, recognizing neurofilament protein was purchased from Zymed, rabbit anti-axonin-1 was described earlier [75]. Secondary antibodies goat anti-mouse Cy3 (Jackson Laboratories) and goat anti-rabbit Alexa488 (Molecular Probes) were used to visualize primary antibody binding sites. Sections were prepared as detailed above. The staining protocol was as detailed earlier [54].

Authors' contributions

OM carried out all the in situ hybridizations and data analyses, RS contributed to probe preparation, tissue sectioning, and immunohistochemistry. JG and MG carried out the bioinformatics analysis, ES conceived and coordinated the study, and wrote the manuscript. Figures were prepared by OM, MG, and ES. All authors read and approved the final manuscript.

Additional material

Additional File 1

Sense controls of stage-18 spinal cord sections. *Adjacent transverse sections of stage-18 spinal cords were hybridized with the respective sense probes for a comparison with the antisense probes shown in Figure 2. Bar 50 μm.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-213X-6-32-S1.pdf]

Additional File 2

Sense controls of stage-22 spinal cord sections. *Transverse sections of stage-22 spinal cords adjacent to the ones shown in Figure 4 were hybridized with the respective sense probes as a negative control4. Bar 50 μm.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-213X-6-32-S2.pdf]

Additional File 3

The expression patterns of plexins and neuropilins do not change significantly within the lumbosacral region of the spinal cord. *Whole-mount preparations of stage 26 spinal cord were used for in situ hybridization to detect plexin and neuropilin mRNAs. At the resolution of whole-mounts no changes were detectable throughout the lumbosacral region of the spinal cord. The only exception being expression levels of npn-2 mRNA that seemed to decrease in some segments of the lumbosacral spinal cord (arrowhead). Bar 500 μm, 200 μm in A-C.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-213X-6-32-S3.pdf]

Acknowledgements

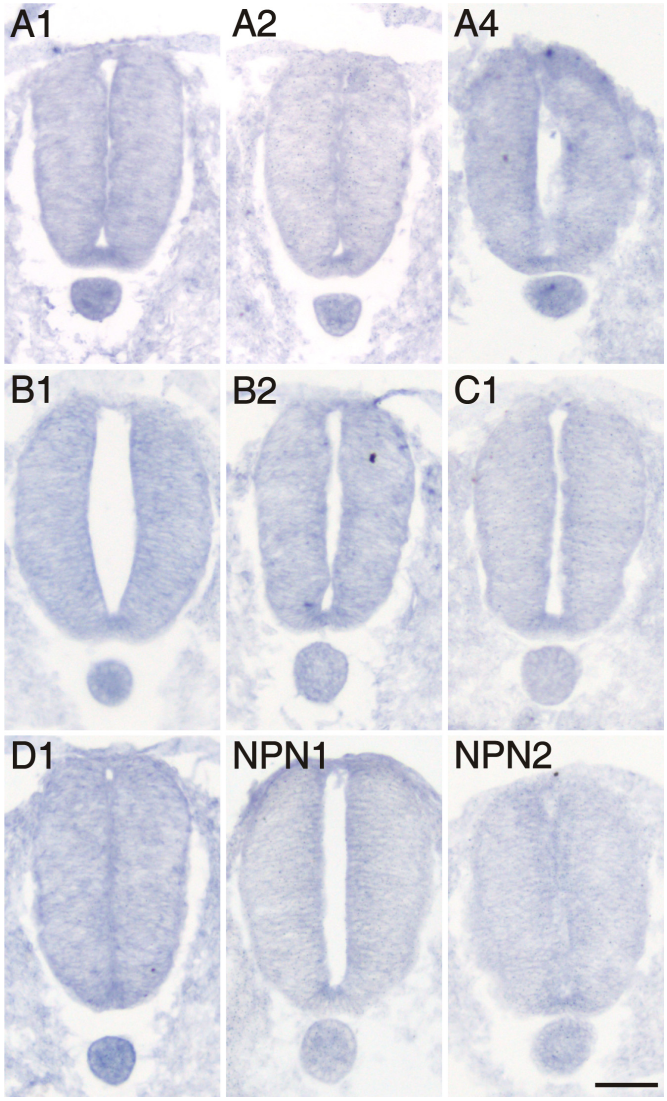
We thank Maja Hess for excellent technical help. Research in the lab of ES is supported by the Swiss National Science Foundation. Research in the lab of MG is supported by grants from the Roche Research Foundation, the Hartmann Müller Stiftung, and the Swiss National Science Foundation.

References

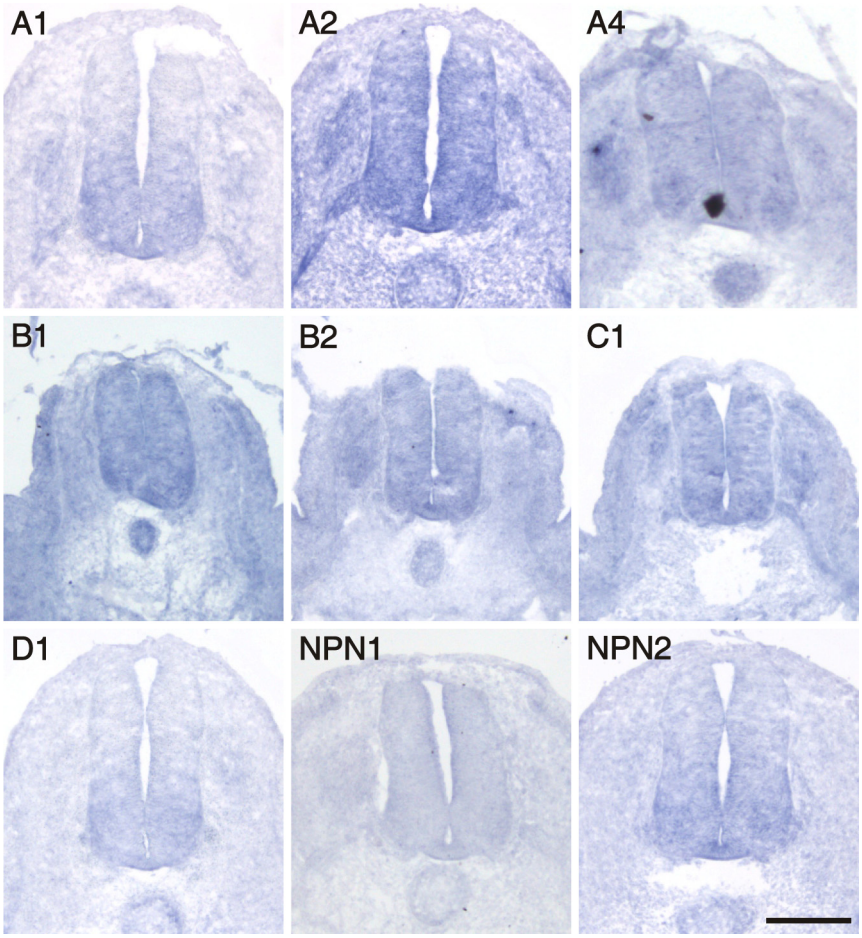
1. Chen H, Chedotal A, He Z, Goodman CS, Tessier-Lavigne M: **Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III.** *Neuron* 1997, **19:**547-59.
2. He Z, Tessier-Lavigne M: **Neuropilin is a receptor for the axonal chemorepellent Semaphorin III.** *Cell* 1997, **90:**739-51.
3. Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD: **Neuropilin is a semaphorin III receptor.** *Cell* 1997, **90:**753-62.
4. Fujisawa H, Kitsukawa T: **Receptors for collapsin/semaphorins.** *Curr Opin Neurobiol* 1998, **8:**587-92.
5. Comeau MR, Johnson R, DuBose RF, Petersen M, Gearing P, VandenBos T, Park L, Farrah T, Buller RM, Cohen JI, *et al.*: **A poxvirus-encoded semaphorin induces cytokine production from monocytes and binds to a novel cellular semaphorin receptor, VESPR.** *Immunity* 1998, **8:**473-82.
6. Winberg ML, Noordermeer JN, Tamagnone L, Comoglio PM, Spriggs MK, Tessier-Lavigne M, Goodman CS: **Plexin A is a neuronal semaphorin receptor that controls axon guidance.** *Cell* 1998, **95:**903-16.
7. Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, Chedotal A, Winberg ML, Goodman CS, Poo M, *et al.*: **Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates.** *Cell* 1999, **99:**71-80.
8. Takahashi T, Fournier A, Nakamura F, Wang LH, Murakami Y, Kalb RG, Fujisawa H, Strittmatter SM: **Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors.** *Cell* 1999, **99:**59-69.
9. Rohm B, Ottemeyer A, Lohrum M, Puschel AW: **Plexin/neuropilin complexes mediate repulsion by the axonal guidance signal semaphorin 3A.** *Mech Dev* 2000, **93:**95-104.
10. Takagi S, Tsuji T, Amagai T, Takamatsu T, Fujisawa H: **Specific cell surface labels in the visual centers of Xenopus laevis tadpole identified using monoclonal antibodies.** *Dev Biol* 1987, **122:**90-100.
11. Ohta K, Mizutani A, Kawakami A, Murakami Y, Kasuya Y, Takagi S, Tanaka H, Fujisawa H: **Plexin: a novel neuronal cell surface molecule that mediates cell adhesion via a homophilic binding mechanism in the presence of calcium ions.** *Neuron* 1995, **14:**1189-99.
12. Satoda M, Takagi S, Ohta K, Hirata T, Fujisawa H: **Differential expression of two cell surface proteins, neuropilin and plexin, in Xenopus olfactory axon subclasses.** *J Neurosci* 1995, **15:**942-55.
13. Fujisawa H: **Discovery of semaphorin receptors, neuropilin and plexin, and their functions in neural development.** *J Neurobiol* 2004, **59:**24-33.
14. Tamagnone L, Comoglio PM: **Signalling by semaphorin receptors: cell guidance and beyond.** *Trends Cell Biol* 2000, **10:**377-83.
15. Murakami Y, Suto F, Shimizu M, Shinoda T, Kameyama T, Fujisawa H: **Differential expression of plexin-A subfamily members in the mouse nervous system.** *Dev Dyn* 2001, **220:**246-58.
16. Fiore R, Puschel AW: **The function of semaphorins during nervous system development.** *Front Biosci* 2003, **8:**s484-99.
17. Huber AB, Kolodkin AL, Ginty DD, Cloutier JF: **Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance.** *Annu Rev Neurosci* 2003, **26:**509-63.
18. Toyofuku T, Zhang H, Kumanogoh A, Takegahara N, Suto F, Kamei J, Aoki K, Yabuki M, Hori M, Fujisawa H, *et al.*: **Dual roles of Sema6D in cardiac morphogenesis through region-specific association of its receptor, Plexin-A1, with off-track and vascular endothelial growth factor receptor type 2.** *Genes Dev* 2004, **18:**435-47.
19. Suto F, Ito K, Uemura M, Shimizu M, Shinkawa Y, Sanbo M, Shinoda T, Tsuboi M, Takashima S, Yagi T, *et al.*: **Plexin-a4 mediates axon-repulsive activities of both secreted and transmembrane semaphorins and plays roles in nerve fiber guidance.** *J Neurosci* 2005, **25:**3628-37.
20. Gu C, Yoshida Y, Livet J, Reimert DV, Mann F, Merte J, Henderson CE, Jessell TM, Kolodkin AL, Ginty DD: **Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins.** *Science* 2005, **307:**265-8.
21. Kolodkin AL, Ginty DD: **Steering clear of semaphorins: neuropilins sound the retreat.** *Neuron* 1997, **19:**1159-62.
22. Nakamura F, Kalb RG, Strittmatter SM: **Molecular basis of semaphorin-mediated axon guidance.** *J Neurobiol* 2000, **44:**219-29.
23. Raper JA: **Semaphorins and their receptors in vertebrates and invertebrates.** *Curr Opin Neurobiol* 2000, **10:**88-94.
24. Castellani V, Rougon G: **Control of semaphorin signaling.** *Curr Opin Neurobiol* 2002, **12:**532-41.
25. Pasterkamp RJ, Kolodkin AL: **Semaphorin junction: making tracks toward neural connectivity.** *Curr Opin Neurobiol* 2003, **13:**79-89.
26. Giordano S, Corso S, Conrotto P, Artigiani S, Gilestro G, Barberis D, Tamagnone L, Comoglio PM: **The semaphorin 4D receptor controls invasive growth by coupling with Met.** *Nat Cell Biol* 2002, **4:**720-4.
27. Toyofuku T, Zhang H, Kumanogoh A, Takegahara N, Yabuki M, Harada K, Hori M, Kikutani H: **Guidance of myocardial patterning in cardiac development by Sema6D reverse signalling.** *Nat Cell Biol* 2004, **6:**1204-11.
28. Neufeld G, Shraga-Heled N, Lange T, Guttmann-Raviv N, Herzog Y, Kessler O: **Semaphorins in cancer.** *Front Biosci* 2005, **10:**751-60.
29. Pasterkamp RJ, Peschon JJ, Spriggs MK, Kolodkin AL: **Semaphorin 7A promotes axon outgrowth through integrins and MAPKs.** *Nature* 2003, **424:**398-405.
30. van der Zwaag B, Hellemons AJ, Leenders WP, Burbach JP, Brunner HG, Padberg GW, Van Bokhoven H: **PLEXIN-D1, a novel plexin family member, is expressed in vascular endothelium and the central nervous system during mouse embryogenesis.** *Dev Dyn* 2002, **225:**336-43.

31. Gitler AD, Lu MM, Epstein JA: **PlexinD1 and semaphorin signaling are required in endothelial cells for cardiovascular development.** *Dev Cell* 2004, **7:**107-16.
32. Torres-Vazquez J, Gitler AD, Fraser SD, Berk JD, Van NP, Fishman MC, Childs S, Epstein JA, Weinstein BM: **Semaphorin-plexin signaling guides patterning of the developing vasculature.** *Dev Cell* 2004, **7:**117-23.
33. Hillier LW, Miller W, Birney E, Warren W, Hardison RC, Ponting CP, Bork P, Burt DW, Groenen MA, Delany ME, *et al.*: **Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution.** *Nature* 2004, **432:**695-716.
34. Smith CA, Sinclair AH: **Sex determination: insights from the chicken.** *Bioessays* 2004, **26:**120-32.
35. Vinogradov AE: **Intron-genome size relationship on a large evolutionary scale.** *J Mol Evol* 1999, **49:**376-84.
36. Hamburger V, Hamilton HL: **A series of normal stages in the development of the chick embryo.** *J Morphol* 1951, **88:**49-92.
37. Helms AW, Johnson JE: **Specification of dorsal spinal cord interneurons.** *Curr Opin Neurobiol* 2003, **13:**42-9.
38. Stoeckli ET, Landmesser LT: **Axonin-1, Nr-CAM, and Ng-CAM play different roles in the in vivo guidance of chick commissural neurons.** *Neuron* 1995, **14:**1165-79.
39. Leonardo ED, Hinck L, Masu M, Keino-Masu K, Fazeli A, Stoeckli ET, Ackerman SL, Weinberg RA, Tessier-Lavigne M: **Guidance of developing axons by netrin-1 and its receptors.** *Cold Spring Harb Symp Quant Biol* 1997, **62:**467-78.
40. Charron F, Stein E, Jeong J, McMahon AP, Tessier-Lavigne M: **The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance.** *Cell* 2003, **113:**11-23.
41. Bourikas D, Pekarik V, Baeriswyl T, Grunditz A, Sadhu R, Nardo M, Stoeckli ET: **Sonic hedgehog guides commissural axons along the longitudinal axis of the spinal cord.** *Nat Neurosci* 2005, **8:**297-304.
42. Landmesser L: **The development of motor projection patterns in the chick hind limb.** *J Physiol* 1978, **284:**391-414.
43. Landmesser LT: **The acquisition of motoneuron subtype identity and motor circuit formation.** *Int J Dev Neurosci* 2001, **19:**175-82.
44. Tsuchida T, Ensini M, Morton SB, Baldassare M, Edlund T, Jessell TM, Pfaff SL: **Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes.** *Cell* 1994, **79:**957-70.
45. Lin JH, Saito T, Anderson DJ, Lance-Jones C, Jessell TM, Arber S: **Functionally related motor neuron pool and muscle sensory afferent subtypes defined by coordinate ETS gene expression.** *Cell* 1998, **95:**393-407.
46. Sharma K, Belmonte JC: **Development of the limb neuromuscular system.** *Curr Opin Cell Biol* 2001, **13:**204-10.
47. Price SR, De Marco Garcia NV, Ranscht B, Jessell TM: **Regulation of motor neuron pool sorting by differential expression of type II cadherins.** *Cell* 2002, **109:**205-16.
48. Tanabe Y, Jessell TM: **Diversity and pattern in the developing spinal cord.** *Science* 1996, **274:**1115-23.
49. Briscoe J, Ericson J: **The specification of neuronal identity by graded Sonic Hedgehog signalling.** *Semin Cell Dev Biol* 1999, **10:**353-62.
50. Jessell TM: **Neuronal specification in the spinal cord: inductive signals and transcriptional codes.** *Nat Rev Genet* 2000, **1:**20-9.
51. Arber S, Ladle DR, Lin JH, Frank E, Jessell TM: **ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons.** *Cell* 2000, **101:**485-98.
52. Livet J, Sigrist M, Stroebel S, De Paola V, Price SR, Henderson CE, Jessell TM, Arber S: **ETS gene Pea3 controls the central position and terminal arborization of specific motor neuron pools.** *Neuron* 2002, **35:**877-92.
53. Eide AL, Glover JC: **Developmental dynamics of functionally specific primary sensory afferent projections in the chicken embryo.** *Anat Embryol (Berl)* 1997, **195:**237-50.
54. Perrin FE, Rathjen FG, Stoeckli ET: **Distinct subpopulations of sensory afferents require F11 or axonin-1 for growth to their target layers within the spinal cord of the chick.** *Neuron* 2001, **30:**707-23.
55. Scott S: **Sensory Neurons: Diversity, Development, and Plasticity.** New York: Oxford University Press; 1992.
56. Cheng HJ, Bagri A, Yaron A, Stein E, Pleasure SJ, Tessier-Lavigne M: **Plexin-A3 mediates semaphorin signaling and regulates the development of hippocampal axonal projections.** *Neuron* 2001, **32:**249-63.
57. Perala NM, Immonen T, Sariola H: **The expression of plexins during mouse embryogenesis.** *Gene Expr Patterns* 2005, **5:**355-62.
58. Brown CB, Feiner L, Lu MM, Li J, Ma X, Webber AL, Jia L, Raper JA, Epstein JA: **PlexinA2 and semaphorin signaling during cardiac neural crest development.** *Development* 2001, **128:**3071-80.
59. Yaron A, Huang PH, Cheng HJ, Tessier-Lavigne M: **Differential requirement for Plexin-A3 and -A4 in mediating responses of sensory and sympathetic neurons to distinct class 3 Semaphorins.** *Neuron* 2005, **45:**513-23.
60. Briscoe J, Pierani A, Jessell TM, Ericson J: **A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube.** *Cell* 2000, **101:**435-45.
61. Wijgerde M, McMahon JA, Rule M, McMahon AP: **A direct requirement for Hedgehog signaling for normal specification of all ventral progenitor domains in the presumptive mammalian spinal cord.** *Genes Dev* 2002, **16:**2849-64.
62. Suto F, Murakami Y, Nakamura F, Goshima Y, Fujisawa H: **Identification and characterization of a novel mouse plexin, plexin-A4.** *Mech Dev* 2003, **120:**385-96.
63. Worzfeld T, Puschel AW, Offermanns S, Kuner R: **Plexin-B family members demonstrate non-redundant expression patterns in the developing mouse nervous system: an anatomical basis for morphogenetic effects of Sema4D during development.** *Eur J Neurosci* 2004, **19:**2622-32.
64. Kawakami A, Kitsukawa T, Takagi S, Fujisawa H: **Developmentally regulated expression of a cell surface protein, neuropilin, in the mouse nervous system.** *J Neurobiol* 1996, **29:**1-17.
65. Chen H, Bagri A, Zupicich JA, Zou Y, Stoeckli E, Pleasure SJ, Lowenstein DH, Skarnes WC, Chedotal A, Tessier-Lavigne M: **Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections.** *Neuron* 2000, **25:**43-56.
66. Zou Y, Stoeckli E, Chen H, Tessier-Lavigne M: **Squeezing axons out of the gray matter: a role for slit and semaphorin proteins from midline and ventral spinal cord.** *Cell* 2000, **102:**363-75.
67. **BBSRC ChickEST database** [http://www.chick.umist.ac.uk]
68. **Ensembl chicken genome database** [http://www.ensembl.org/Multi/blastview?species=Gallus_gallus]
69. **Genscan gene prediction program** [http://genes.mit.edu/GENSCAN.html]
70. **SMART program** [http://smart.embl-heidelberg.de]
71. Higgins DG, Sharp PM: **Fast and sensitive multiple sequence alignments on a microcomputer.** *Comput Appl Biosci* 1989, **5:**151-3.
72. Thompson JD, Higgins DG, Gibson TJ: **CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.** *Nucleic Acids Res* 1994, **22:**4673-80.
73. Page RD: **TreeView: an application to display phylogenetic trees on personal computers.** *Comput Appl Biosci* 1996, **12:**357-8.
74. **NCBI-BLAST program** [http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi]
75. Stoeckli ET, Kuhn TB, Duc CO, Ruegg MA, Sonderegger P: **The axonally secreted protein axonin-1 is a potent substratum for neurite growth.** *J Cell Biol* 1991, **112:**449-55.

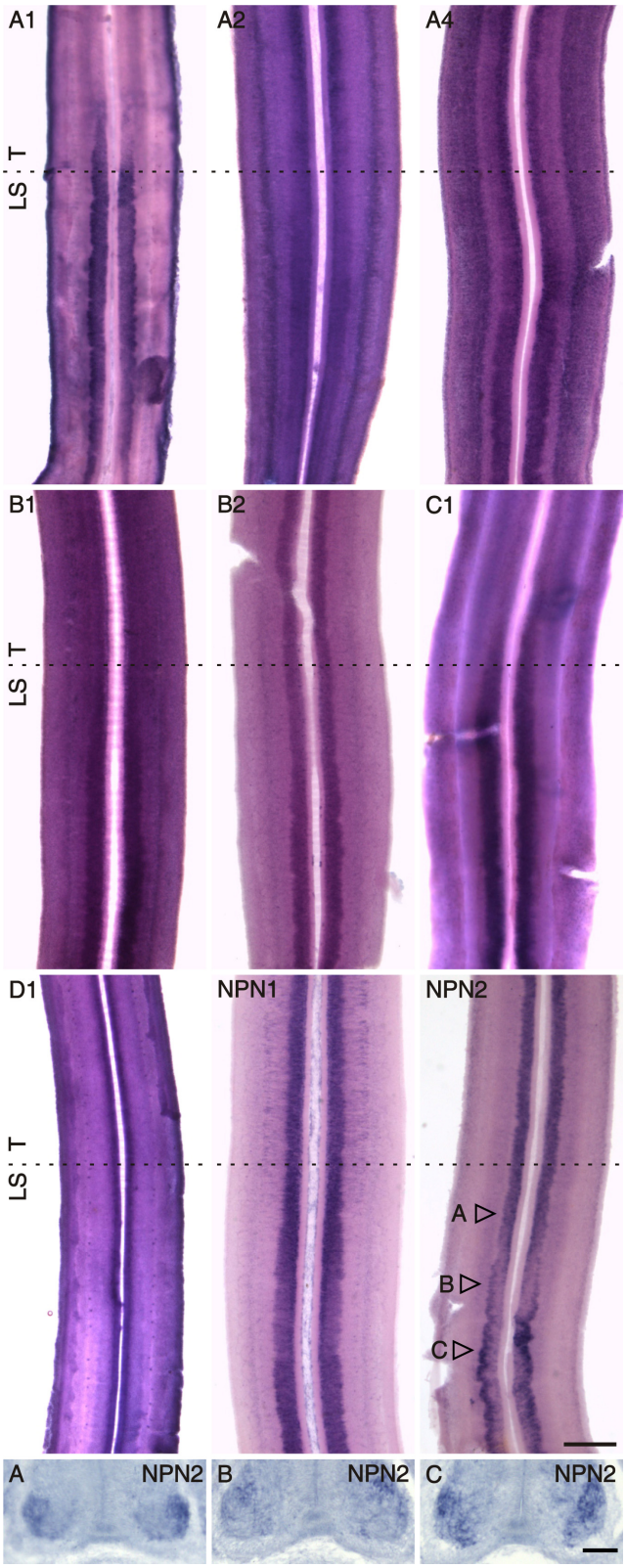
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



5. Functional Analysis of Semaphorin6A

Semaphorin6A acts as a gate keeper between the central and the peripheral nervous system

Olivier Mauti, Elena Domanitskaya, Irwin Andermatt, Rejina Sadhu, and Esther T. Stoeckli

Institute of Zoology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

Published in Neural Development, 2007, 2:28

This paper was evaluated by the Faculty of 1000 Biology.

Neural Development

BioMed Central

Research article

Open Access

Semaphorin6A acts as a gate keeper between the central and the peripheral nervous system

Olivier Mauti[1], Elena Domanitskaya[1], Irwin Andermatt[1], Rejina Sadhu[1,2] and Esther T Stoeckli*[1]

Address: [1]Developmental Neuroscience, Institute of Zoology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland and [2]Novartis, DOC, Lichtstrasse 35, CH-4002 Basel, Switzerland

Email: Olivier Mauti - Olivier.Mauti@zool.uzh.ch; Elena Domanitskaya - Elena.Domanitskaya@zool.uzh.ch; Irwin Andermatt - Irwin.Andermatt@zool.uzh.ch; Rejina Sadhu - rejina.sadhu@novartis.com; Esther T Stoeckli* - Esther.Stoeckli@zool.uzh.ch

* Corresponding author

Published: 18 December 2007

Neural Development 2007, **2**:28 doi:10.1186/1749-8104-2-28

Received: 2 August 2007
Accepted: 18 December 2007

This article is available from: http://www.neuraldevelopment.com/content/2/1/28

Abstract

Background: During spinal cord development, expression of chicken SEMAPHORIN6A (SEMA6A) is almost exclusively found in the boundary caps at the ventral motor axon exit point and at the dorsal root entry site. The boundary cap cells are derived from a population of late migrating neural crest cells. They form a transient structure at the transition zone between the peripheral nervous system (PNS) and the central nervous system (CNS). Ablation of the boundary cap resulted in emigration of motoneurons from the ventral spinal cord along the ventral roots. Based on its very restricted expression in boundary cap cells, we tested for a role of Sema6A as a gate keeper between the CNS and the PNS.

Results: Downregulation of Sema6A in boundary cap cells by *in ovo* RNA interference resulted in motoneurons streaming out of the spinal cord along the ventral roots, and in the failure of dorsal roots to form and segregate properly. PlexinAs interact with class 6 semaphorins and are expressed by both motoneurons and sensory neurons. Knockdown of PlexinA1 reproduced the phenotype seen after loss of Sema6A function both at the ventral motor exit point and at the dorsal root entry site of the lumbosacral spinal cord. Loss of either PlexinA4 or Sema6D function had an effect only at the dorsal root entry site but not at the ventral motor axon exit point.

Conclusion: Sema6A acts as a gate keeper between the PNS and the CNS both ventrally and dorsally. It is required for the clustering of boundary cap cells at the PNS/CNS interface and, thus, prevents motoneurons from streaming out of the ventral spinal cord. At the dorsal root entry site it organizes the segregation of dorsal roots.

Background

During development of the nervous system, axons navigate long distances to connect to their targets. Along their trajectories they encounter a large variety of guidance cues that support their navigation [1,2]. Axon guidance cues are subdivided into long-range and short-range guidance cues. They belong to a relatively small number of protein families, the immunoglobulin superfamily of cell adhesion molecules [3], the Eph/ephrins [4], the netrins [5,6], the semaphorins [7,8] and their receptors, plexins and

neuropilins [9,10]. More recently, morphogens such as Wnts and Shh have also been implicated in axon guidance [11-13].

The semaphorins comprise a large family subdivided into eight subclasses based on structural criteria and their expression in vertebrates or non-vertebrate organisms [7,14,15]. Class 1 and 2 semaphorins are expressed only in invertebrates, classes 3, 4, 6, and 7 are expressed only in vertebrates, class 5 semaphorins are expressed in both invertebrates and vertebrates, whereas class V consists of a viral semaphorin. With respect to their function, soluble class 3 semaphorins are the best characterized. They have been shown to act mainly as repellents but, in some cases, also as attractants for extending axons. Class 3 semaphorins bind to a receptor complex composed of Neuropilin-1 or -2 and a member of the class A plexins [10,15], although there is at least one exception to this rule [16].

Plexins are expressed in a highly dynamic pattern during development of the nervous system [17-20]. They are subdivided into four classes comprising a total of nine members in mammals and seven members in chicken [20]. Plexins of class A and B were shown to bind to transmembrane semaphorins in the absence of neuropilins [21,22]. PlexinBs are receptors for class 4 semaphorins, whereas PlexinAs were shown to be receptors for class 6 semaphorins [22-25]. Interestingly, transmembrane semaphorins have functions in axon guidance and synapse formation that are independent of neuropilins [22]. The long cytoplasmic tail of Sema6A contains binding sites for Ena/VASP-like protein EVL and may, therefore, directly regulate cytoskeletal dynamics [26]. Consistent with these structural features, Sema6A was suggested to act as a receptor [27], similar to findings for Sema1a, the closest ortholog of Sema6A in invertebrates [28]. Sema1a was shown to act both as a repellent [29,30] and as an attractant [31]. A receptor function for Sema1a was reported in the visual system of *Drosophila*, where photoreceptor cells depended on Sema1a for their targeting to the optic lobe [32].

In mammals, Sema6A was shown to affect pathfinding of thalamocortical axons [27] and to be required for cell migration in the cerebellum [33]. The mode of action has not been determined in these studies but, based on the expression pattern and the analysis of the phenotypes, a repulsive mechanism has been suggested in the latter. This would be consistent with *in vitro* studies that demonstrated a repulsive role of Sema6A on sympathetic axons [22,34]. More recently, a repellent activity of Sema6D on proprioceptive sensory afferents has been shown in both mouse and chicken [25]. The targeting of proprioceptive axons was dependent on PlexinA1 mediating the repulsive activity of Sema6C/D. PlexinA1 was also shown to be the binding partner of Sema6D in neural crest cell migration during heart development [23]. In these studies a receptor function of Sema6D was demonstrated [24]. Thus, transmembrane class 6 semaphorins are bifunctional molecules in axon guidance and cell migration. They can act as a ligand for PlexinAs but also transmit a signal themselves.

In vertebrates, the receptors for Sema6A in cerebellar development have not been identified. However, *in vitro* binding studies have indicated that Sema6A can bind to PlexinA2 and A4 [22], whereas Sema6B binds to PlexinA1and A4 [22], Sema6C was suggested to bind to PlexinD1 [35], and finally Sema6D was shown to bind to PlexinA1 in neural crest cell migration [23,24].

Analysis of SEMA6A expression during chicken spinal cord development revealed its restriction to the ventral ventricular zone, the origin of oligodendrocytes, and, most strikingly, to cells at the ventral motor axon exit point (VMEP) and the dorsal sensory axons entry point. Cells located at the transition zone between the PNS and the CNS were shown to have gate keeper function [36,37]. In analogy to their function they are called boundary cap cells (BCCs). BCCs are derived from a late migrating population of neural crest cells [38]. They express Krox20 and the 1E8 antigen in addition to the more general neural crest marker Sox10. BCCs are necessary to prevent emigration of motoneurons from the ventral spinal cord [37]. More recently, the boundary cap was identified as a source of neural crest stem cells that give rise to glia and sensory neurons of the dorsal root ganglion (DRG) [39-41].

Here, we show that Sema6A is required for the gate keeper function of BCCs, as in the absence of Sema6A BCCs fail to cluster properly at the CNS/PNS interface and, thus, cannot prevent the emigration of motoneurons in a PlexinA1-dependent manner. At the dorsal root entry site Sema6A is required for the appropriate segregation of dorsal roots.

Results

SEMA6A is expressed in boundary cap cells

SEMA6A shows a much more restricted expression pattern during development of the chicken spinal cord compared to the mouse. In contrast to the mouse, where SEMA6A was found throughout the ventral spinal cord and in DRGs [22], it was expressed only transiently in the ventral spinal cord but never in DRGs in the chick. Most striking, however, was its expression in cells at the boundary between the CNS and the PNS (Figure 1). SEMA6A was detectable in a ventral stream of neural crest cells at stage 19 (HH19; Figure 1a) [42]. At that time, small clusters of BCCs identified by KROX20 [43] were seen only at the

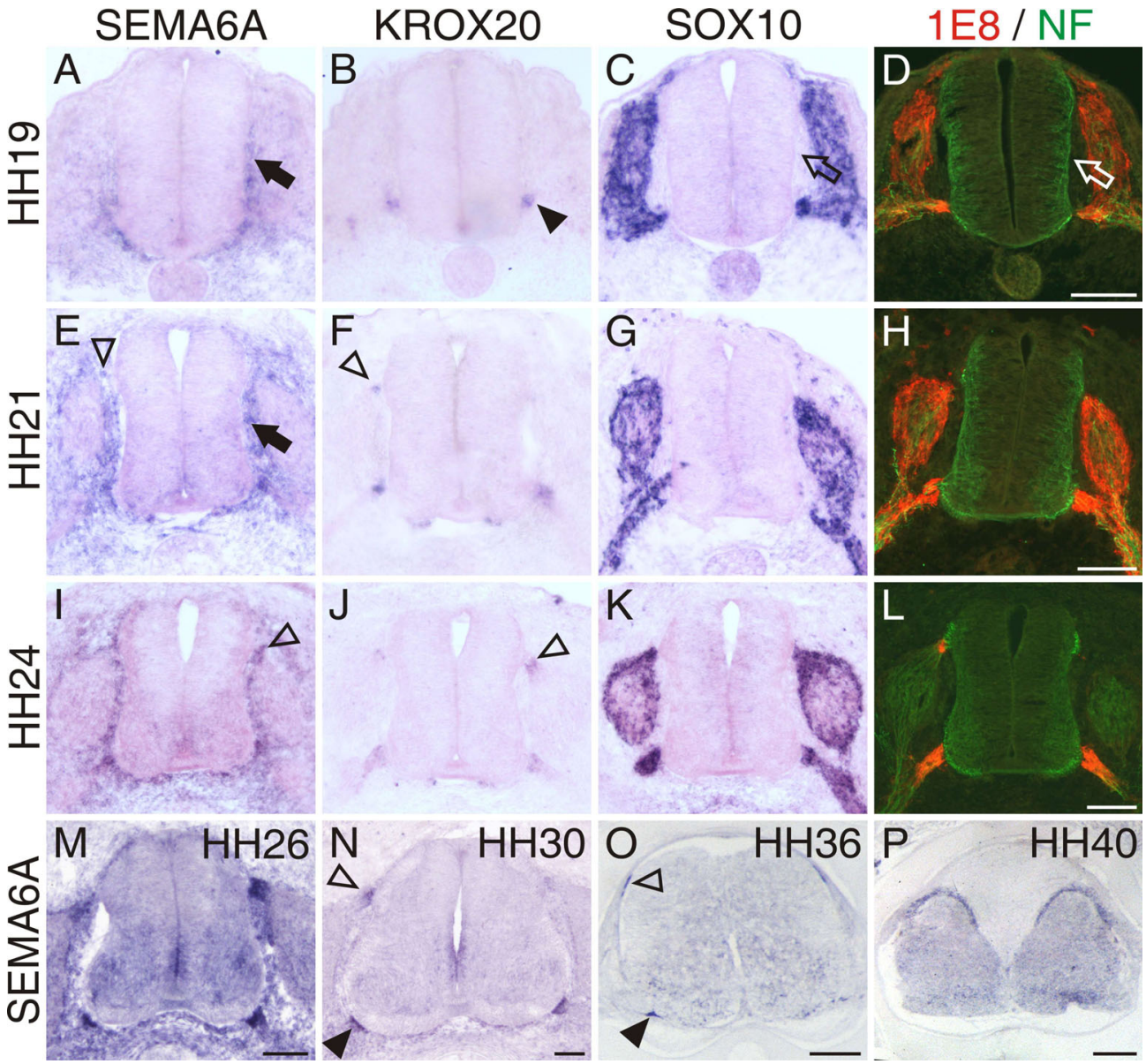


Figure 1

SEMA6A is expressed in neural crest cells that give rise to boundary cap cells. **(a)** Neural crest cells that give rise to boundary cap cells express SEMA6A while they are still migrating ventrally (HH19; arrow). Boundary cap cells start to cluster first at the VMEP. **(b)** Only those cells that have formed clusters at the VMEP express the BCC marker KROX20 (arrowhead). The neural crest markers **(c)** SOX10 and the **(d)** 1E8 epitope are expressed by many neural crest-derived cell populations and are not restricted to BCCs at HH19. Note that neither SOX10 (open arrow in (c)), nor 1E8 (open arrow in (d)) are expressed in BCCs while they are still migrating. First clusters of BCCs next to the DREZ marked by **(e)** SEMA6A expression (open arrowhead) or **(f)** KROX20 (open arrowhead) are detectable at lumbosacral levels by HH21. At this stage, many SEMA6A-expressing cells are still migrating along the neural tube to reach the ventral BCC cluster (arrow in (e)). **(i-l)** A similar situation is found at HH24. SEMA6A expression is clearly detectable in dorsal BCCs (open arrowhead in (i); compare with (j)). **(n, o)** After HH30, SEMA6A expression in dorsal (open arrowhead in (n)) and ventral (arrowhead in (n)) BCCs decreased but was still visible by HH36 (o). *In situ* hybridizations on adjacent transverse sections of the lumbosacral spinal cord are shown for SEMA6A (a, e, i, m-p), KROX20 (b, f, j), and SOX10 (c, g, k) at HH19 (a-d), HH21 (e-h), HH24 (i-l) as indicated. Sections shown in (d, h, l) were stained for 1E8 (red) and neurofilament (green). Bars are 100 μm in (a-n), 200 μm in (o), and 500 μm in (p).

VMEP (Figure 1b). Motor axons start to leave the spinal cord shortly before the cluster of BCCs is detectable by 1E8 staining (data not shown) [37,44]. Clustering of BCCs at the dorsal root entry site started at HH21, as visualized by KROX20 (Figure 1f). By HH24, BCC clusters were very prominent both ventrally at the VMEP and dorsally at the dorsal root entry zone (DREZ; Figure 1j). BCCs expressed SEMA6A (Figure 1a,e,i,m–o), KROX20 (Figure 1b,f,j), SOX10 (Figure 1c,g,k) [45], the 1E8 antigen (an epitope of P0; Figure 1d,h,l) [46], and Cadherin-7 (data not shown) [47]. The SEMA6A-expressing cells were not SOX10 or 1E8 positive while they migrated toward the VMEP. Similarly, the expression of KROX20 was visible only after cells had clustered. After clustering, boundary cap cells were positive for SOX10 (Figure 1c,g,k) and 1E8 (Figure 1d,h,l). SOX10 and 1E8 were not restricted to BCCs but were also expressed by Schwann cells associated with the ventral roots and in DRGs. Thus, SEMA6A is the earliest marker for cells that end up in clusters at the boundary between the CNS and the PNS.

Sema6A is required to keep motoneurons from migrating out of the ventral spinal cord

BCCs at the VMEP were shown to prevent the emigration of motoneurons from the ventral spinal cord [37]. The failure in BCC cluster formation after ablation of neural crest cells resulted in streams of motoneurons migrating along the axons of the ventral root. Because of the restricted expression of SEMA6A in BCCs, we set out to test whether Sema6A would be required for the role of BCCs as gate keepers between the CNS and the PNS. To this end, we used *in ovo* RNA interference (RNAi), our previously established method to induce loss-of-function phenotypes [48]. *In ovo* RNAi at HH12-14 efficiently targeted neural crest cells and resulted in downregulation of Sema6A but did not interfere with the expression of other family members of class 6 semaphorins (data not shown). Downregulation of Sema6A did indeed reproduce the phenotype seen after ablation of the BCCs (Figure 2) [37]. Groups of motoneurons identified by Isl-1 staining were found along the ventral roots in all HH25 embryos lacking Sema6A function (Figure 2a). Motoneurons exiting the spinal cord were seen, on average, in 40% of the sections from the lumbosacral spinal cord (range 25–54%). Single motoneurons leaving the ventral spinal cord were occasionally detected in control embryos (Figure 2b). However, cells did not emigrate in clusters as seen after downregulation of Sema6A, and the number of sections that contained motoneurons along the ventral roots was much smaller in control-treated embryos compared to embryos lacking Sema6A function. Downregulation of the other class 6 semaphorins, Sema6B (12%) and Sema6D (10%), did not significantly enhance emigration of motoneurons compared to control embryos expressing enhanced green fluorescent protein (EGFP; 8%). SEMA6D but not SEMA6B was found to be expressed in BCCs (IA and ES, unpublished observation). However, as seen for KROX20, expression started only after clustering of BCCs. SEMA6D was not found in BCCs that were still migrating (data not shown).

Sema6A is required for appropriate entry of sensory afferents into the dorsal spinal cord

The strong effect on motoneurons and the fact that SEMA6A was expressed also in BCCs at the DREZ prompted us to analyze the effect of Sema6A downregula-

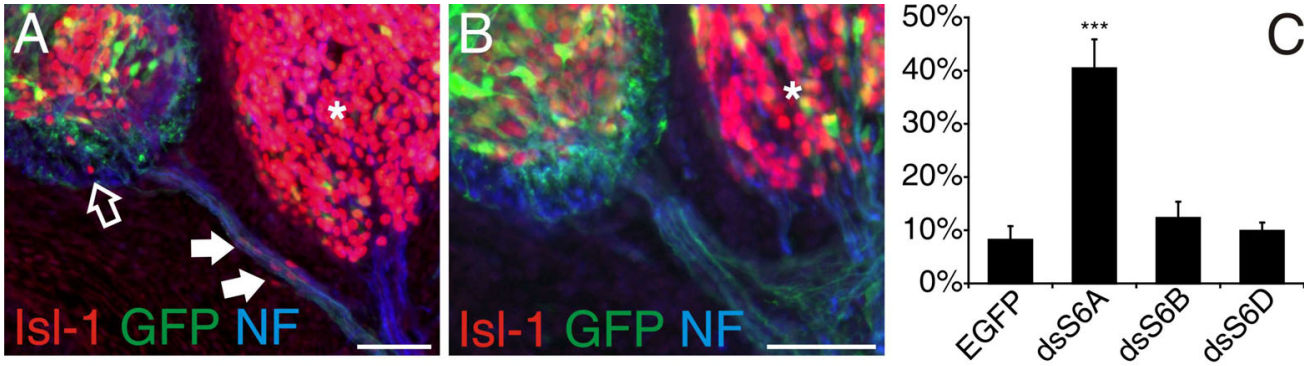


Figure 2

Downregulation of Sema6A in BCCs results in translocation of motoneurons out of the spinal cord. **(a)** In the absence of Sema6A from BCCs, motoneurons stream out of the ventral spinal cord and migrate along the ventral roots (arrows). The open arrow points to a motoneuron that is located in the ventral funiculus. **(b)** In control-treated embryos motoneurons along ventral roots or in the ventral funiculus were rarely seen. Motoneurons were identified by Isl-1 (red). An EGFP plasmid was co-injected with the dsRNA derived from SEMA6A. Axons were stained with an antibody against neurofilament (blue). Note that sensory neurons in the DRG (asterisk in (a, b)) are also stained by Isl-1. **(c)** Perturbation of Sema6B or Sema6D did not enhance the number of motoneurons in the periphery compared to control-treated embryos injected only with the plasmid encoding EGFP. Three asterisks indicate $P < 0.0001$ for the comparison between dsS6A and all other treatment groups. Values are given as mean ± standard error of mean. Bar: 50 μm.

tion on sensory afferents. Loss of Sema6A in dorsal BCCs had a severe effect on the arrangement of dorsal roots (Figure 3). In control embryos analyzed at HH25/26, fibers emanating from a single DRG formed, on average, four to five well separated fiber bundles or roots that entered the dorsal spinal cord via the DREZ. Roots derived from neighboring DRGs were clearly segregated (Figure 3a). This was not the case after downregulation of Sema6A in dorsal BCCs (Figure 3b). In 71% of these embryos the arrangement of dorsal roots and their number were severely perturbed (Figure 3d). Furthermore, the shape of the DRGs was more variable than in control embryos, including many DRGs with a bell shape; that is, with a distance between the most anterior and the most posterior fiber entering the spinal cord that was larger than the anteroposterior size of the DRG (Figure 3e). In control embryos these two lengths were identical, resulting in an arc-like shape of the DRG. In addition to the embryos exhibiting a strong phenotype, we found 18% with a weak phenotype (Figure 3d). In these embryos no bell-shaped DRGs were found despite the fact that the number and arrangement of roots varied. In more than 70% of the embryos lacking Sema6A in BCCs, we found no segregation between adjacent DRGs; that is, roots were formed by fibers emanating from two adjacent DRGs. Only 12% of the embryos treated with double-stranded RNA (dsRNA) derived from SEMA6A were normal. In 58% of the control-treated embryos, DRGs and their roots were normal (Figure 3a,d). Only 13% of them exhibited a strong phenotype.

Interestingly, in contrast to our findings at the VMEP, downregulation of Sema6D resulted in a dorsal phenotype (Figure 3c,d). Embryos lacking Sema6D were, overall, not much different from embryos lacking Sema6A. In only 9% of the embryos were arrangement and number of dorsal roots normal. Sixty-eight percent of the embryos exhibited a strong phenotype, and 23% a weak phenotype. Downregulation of Sema6B resulted in a qualitatively different phenotype. Despite the fact that DRGs exhibited a mushroom-like shape (Figure 3e), the number and the arrangement of the roots were much less affected (data not shown; Figure 3d).

PlexinAs, known receptors for Sema6A, are expressed by motoneurons and sensory neurons

PlexinAs were shown to act both as ligands and as receptors for class 6 semaphorins [23-25]. Previously, we had shown that the expression patterns of chicken PLEXINAs were highly dynamic both in motoneurons and sensory neurons [20]. Based on these analyses, all PlexinAs were potential binding partners for Sema6A. PLEXINA1 was expressed at high levels in the ventral spinal cord and in DRGs at HH18 to HH22 [20] (Figure 4). PLEXINA2 was expressed in the lumbosacral spinal cord at HH18 but was subsequently downregulated in motoneurons during development. Expression in DRGs was weak between HH20 and HH30. In contrast, PLEXINA4 was virtually not expressed in the spinal cord at HH18 but was strongly upregulated in motoneurons at HH22. PLEXINA4 was also expressed in DRGs at HH22 and later stages [20]. Based on the temporal and spatial expression pattern, none of the PlexinAs could be ruled out as a binding partner for Sema6A at the VMEP and at the dorsal root entry site. PLEXINA2 was the least likely candidate because we focused our analysis on the lumbosacral level of the spinal cord, where PLEXINA2 was already below detection levels by HH20, in contrast to the thoracic level where PLEXINA2 remained expressed.

We first knocked down PlexinAs in motoneurons using *in ovo* RNAi. For each PlexinA we used two independent long dsRNAs (see Materials and methods). Downregulation was specific for the targeted gene (Additional file 1). Consistent with its strong expression in motoneurons at the time when they extend their axons out of the VMEP, we found pronounced effects after downregulation of PlexinA1. In 34% of the sections from the lumbosacral region that we analyzed we found groups of motoneurons along the ventral root (Figure 5). All embryos lacking PlexinA1 were affected and had motoneurons outside the spinal cord in 13–52% of the sections taken from the lumbosacral spinal cord. Thus, the phenotype observed after RNAi for PLEXINA1 was qualitatively and quantitatively comparable to the phenotype observed after RNAi for SEMA6A (compare Figures 5a,b,e and 2a,c). Downregulation of PlexinA2 and A4 had no effect on the migratory behavior of motoneurons. The number of motoneurons outside the spinal cord was not different from control (Figure 5e). We counted ectopic motoneurons in 9% of the lumbosacral sections from embryos lacking PlexinA2 or PlexinA4 compared to 8% for control-treated embryos.

Next we analyzed the effect of PlexinA downregulation at the dorsal root entry site. In the absence of PlexinA1 and PlexinA4 (Figure 5c,d), we found phenotypes that resembled those seen after downregulation of Sema6A and Sema6D (Figure 3b,c). Downregulation of PlexinA1 perturbed dorsal root formation and segregation in the vast majority of the embryos. Only 17% of the embryos had normal DRGs (Figure 5f). Seventy percent of them exhibited a strong phenotype. Detailed analysis of the embryos lacking PlexinA1 revealed that the phenotype was qualitatively different from the phenotype seen in the absence of Sema6A. In addition to fusions of adjacent DRGs, we found a different type of DRG shape to predominate in embryos lacking PlexinA1. DRGs were narrower than normal and had a reduced number of roots. The distance between the most anterior and the most posterior fiber emanating from a single DRG was much shorter than the

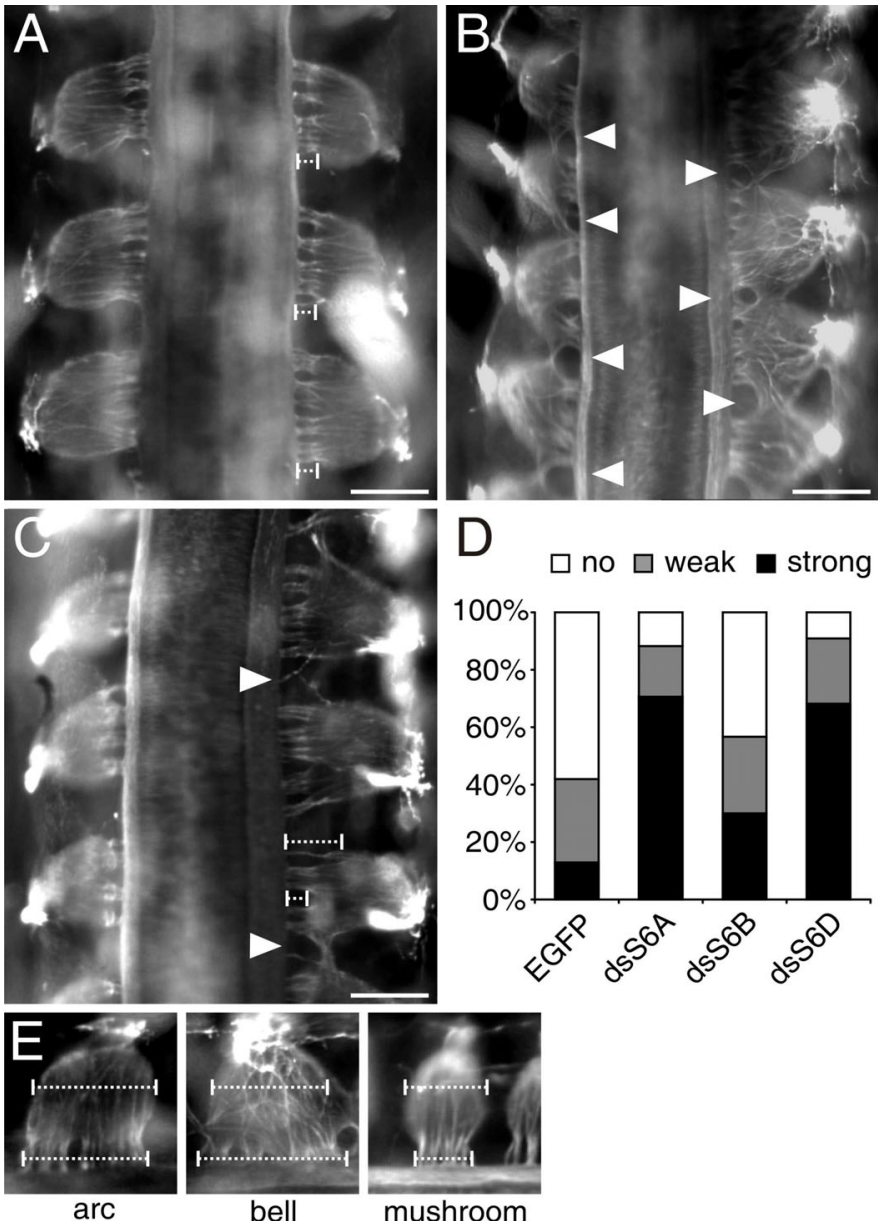


Figure 3

Lack of Sema6A and Sema6D in dorsal BCCs results in aberrant segregation of dorsal roots. **(a)** In control embryos axon bundles from each dorsal root ganglion extend to the DREZ in a well organized manner. Roots from adjacent DRGs are segregated and they are all of the same length (dashed bars). **(b)** In contrast, in embryos lacking Sema6A, roots from adjacent DRGs are no longer segregated (arrowheads). The arrangement of roots arising from individual DRGs is strongly disorganized and roots are often formed by fibers from two adjacent DRGs (arrowheads in (b)). **(c)** Similarly, roots are disorganized in embryos lacking Sema6D (arrowheads). In addition the length of the roots varied more in the absence of Sema6D (compare dashed bars in (c)). **(d)** Strong phenotypes were seen in 71% of the embryos lacking Sema6A and in 68% of the embryos lacking Sema6D. Only 13% of the embryos injected with an EGFP plasmid had a comparable phenotype. Downregulation of Sema6B resulted in aberrant DRG shapes and root arrangement in 30% of the embryos. **(e)** The shapes of DRGs were classified as arc-like when the distance between the most anterior and the most posterior fiber emanating from the DRG was the same as the anteroposterior diameter of the DRG; as bell-shaped when the fibers spread an anteroposterior length that was bigger than the diameter of the DRG; and as mushroom-like when the fibers entered the dorsal spinal cord in a segment that was shorter than the diameter of the DRG. Note that the diameter of the mushroom-like DRGs was smaller than the diameter of arc-like or bell-shaped DRGs. Bar: 200 μm.

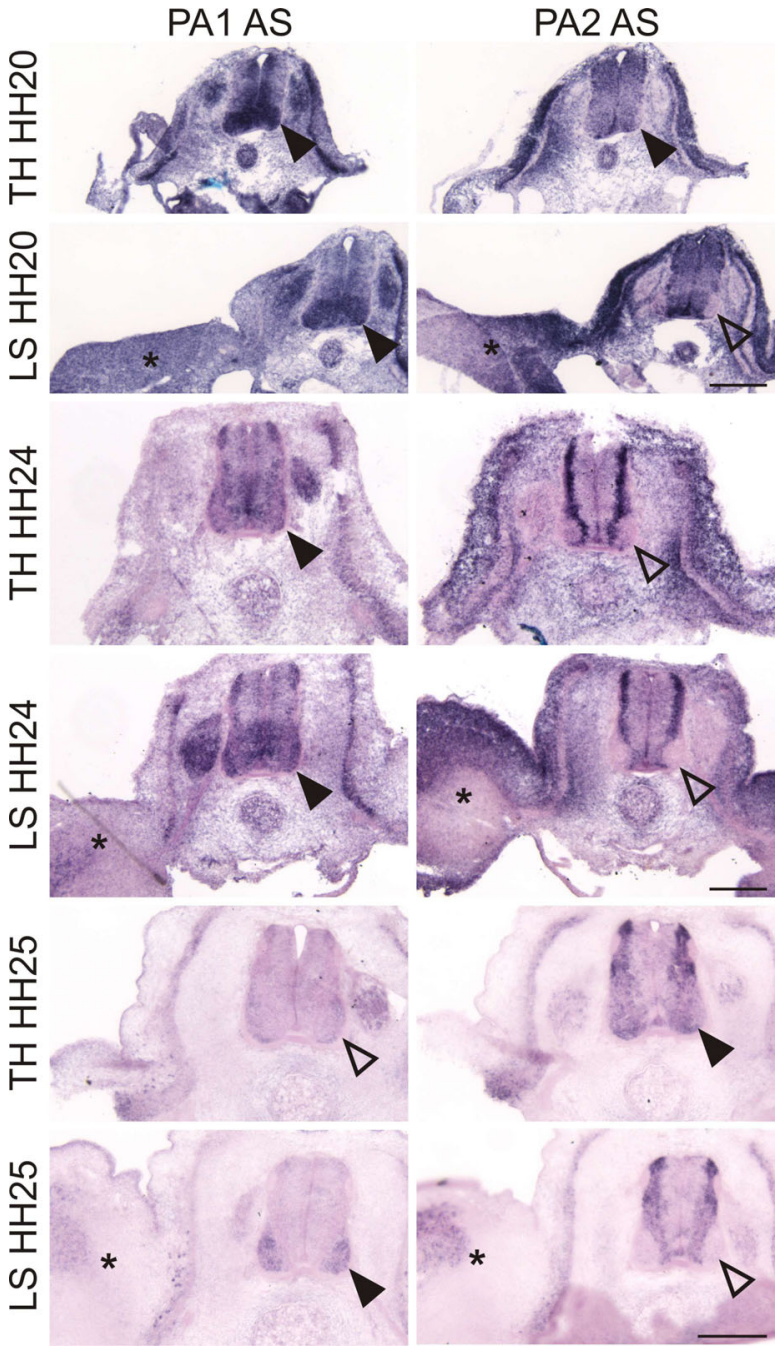


Figure 4
The expression of PLEXINA1 (PA1) and PLEXINA2 (PA2) differs between the thoracic and the lumbosacral levels of the spinal cord. Based on their expression pattern, none of the PlexinAs can be excluded as a binding partner for Sema6A [20]. In addition to the dynamic changes over time, the expression of PLEXINA1 and PLEXINA2 differs strongly between thoracic and lumbosacral levels of the spinal cord. PLEXINA1 is strongly expressed in the ventral spinal cord at HH20, but remains to be expressed strongly only at the lumbosacral but not the thoracic level at HH24 and HH25. Even more pronounced are the changes of PLEXINA2 expression. At HH20, expression is detectable in lateral motoneurons only at the thoracic but not at the lumbosacral level of the spinal cord. This difference is even more pronounced at older stages. AS, antisense probe; TH, thoracic level; LS lumbosacral level. Arrowheads indicate expression of either PA1 or PA2; open arrowheads indicate no or very weak expression. Asterisks label the hind limb to indicate that sections were taken from the lumbosacral level of the spinal cord. Bar: 200 μm.

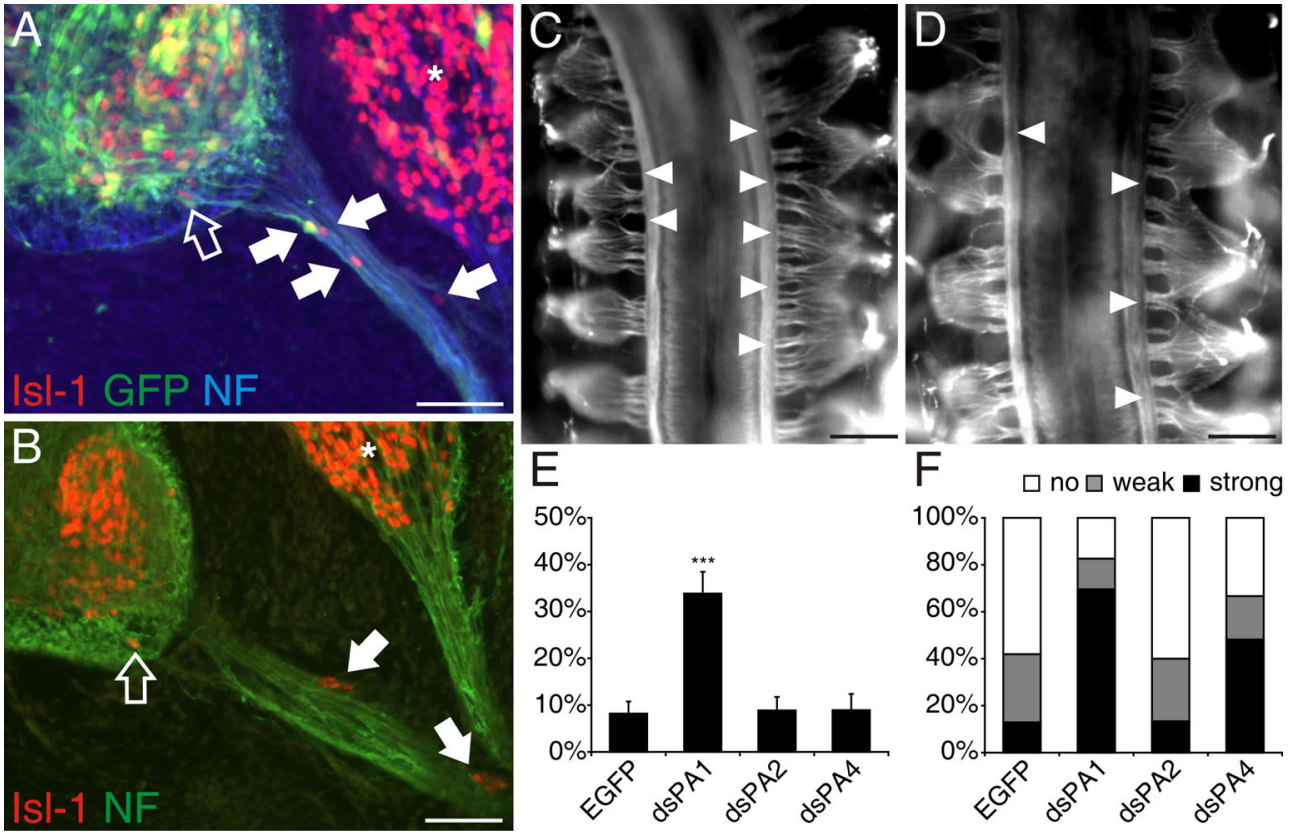


Figure 5
Downregulation of PlexinA1 results in the same phenotype as seen in the absence of Sema6A. **(a, b)** Motoneurons streaming out of the ventral spinal cord identified by Isl-1 staining were only found after downregulation of PlexinA1 (arrows). The open arrow points to a motoneuron that is located in the ventral funiculus. Note that sensory neurons in the DRG (asterisk) are also stained by Isl-1. **(e)** Lack of none of the other PlexinAs enhanced the number of motoneurons found along the ventral roots compared to control-treated embryos (p = 0.0001 for the comparison between dsPA1 and all other treatment groups (indicated by three asterisks); values are given as mean ± standard error of the mean; see Figure 2b). **(c, d)** The phenotype seen after downregulation of Sema6A in dorsal BCCs was mimicked by both lack of PlexinA1 (c) and PlexinA4 (d). The effects of PlexinA downregulation were qualitatively different, however. In the absence of PlexinAs, the arrangement of DRGs, and not only the arrangement of their roots, was disorganized. **(f)** A phenotype was seen in 83% of embryos lacking PlexinA1 and in 67% of the embryos lacking PlexinA4. Bar 50 μm in (a, b); 200 μm in (c, d).

width of the DRG. Therefore, we qualified these DRGs as mushroom-like (Figure 3e). Variable shapes of DRGs were found after loss of PlexinA4 function, where 48% of the embryos exhibited a strong phenotype. In both cases it was sometimes not possible to identify individual DRGs, as they were fused across spinal cord segments. In the absence of PlexinA1, only 17% of the embryos had normal DRGs, and in the absence of PlexinA4, only 33% had normal DRGs. Downregulation of PlexinA2 did not show an effect on dorsal root arrangement; 60% of the embryos were normal. Aberrant root arrangement and mushroom-shaped DRGs were only found in 13% of the embryos.

Sensory but not motor axons are repelled by Sema6A

To get a lead on the mechanism of Sema6A function in boundary control, we turned to an *in vitro* assay (Figure 6). We wanted to assess whether Sema6A in BCCs had an attractive or a repulsive effect on sensory and motor axons, respectively. For this purpose, we transfected COS cells with SEMA6A and used them as a substrate for DRG neurons and motoneurons. We also used sympathetic neurons as they were shown to react to Sema6A contact with growth cone collapse [34]. Axonin-1 was used as a control protein. We scored the behavior of axons encountering transfected COS cells as 'repulsion' when axon failed to grow onto a transfected cell by either stopping or turning away. The score was 'attraction' when axons readily crossed from a non-transfected to a transfected COS cell but did not cross back from the transfected to a non-transfected cell. Axons that readily crossed from a non-transfected to a transfected cell and back to a non-transfected cell were scored as 'crossing', or, in other words, were considered not to be affected by the protein expressed on COS cells. COS cells expressing EGFP were

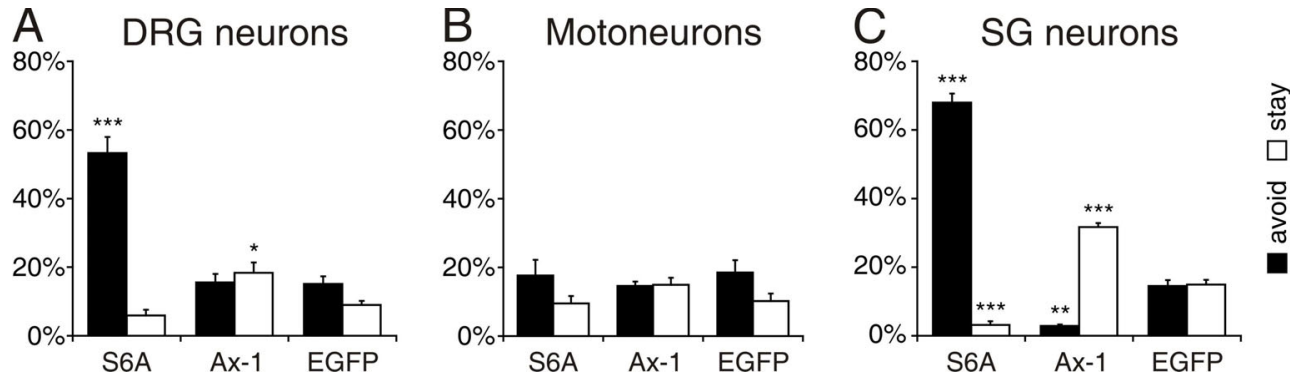


Figure 6

Axons of DRG and sympathetic neurons but not motor axons are repelled by Sema6A. **(a)** Upon encountering a COS cell expressing Sema6A, 53% of all DRG axons were found to react with avoidance, that is, they turned away from the cell or stopped rather than growing onto the Sema6A-positive COS cell ($p = 0.0002$ for the comparison between S6A/Ax-1 and S6A/EGFP (indicated by three asterisks)). COS cells expressing Axonin-1 (Ax-1) were perceived as slightly more attractive than control COS cells expressing only EGFP ($p = 0.006$ for S6A/Ax-1 and 0.02 for S6A/EGFP (indicated by asterisk)). **(b)** Motor axons were found to be indifferent to all types of COS cells. The majority showed neither attraction nor repulsion when encountering Sema6A or Axonin-1 compared to EGFP-expressing COS cells. **(c)** The majority of sympathetic axons (68%) were avoiding Sema6A-expressing cells ($p < 0.0001$ (three asterisks)). Compared to DRG axons and motor axons, sympathetic axons were more strongly attracted by Axonin-1-expressing COS cells ($p < 0.0001$ for Ax-1/S6A and Ax-1/EGFP). This is reflected by the fact that avoidance of Axonin-1-expressing cells was significantly lower compared to EGFP-expressing cells ($p = 0.003$ (two asterisks)). Similarly, axons of sympathetic neurons (SG) were significantly less attracted to Sema6A-expressing compared to EGFP-expressing cells ($p < 0.0001$ (three asterisks)). Values are given as mean ± standard error of the mean.

used as an additional control to measure the 'baseline behavior' of axons growing on COS cells. As expected, few cells reacted with repulsion or attraction to COS cells expressing EGFP. For all types of neurons, we found that more than 70% of the axons crossed EGFP-transfected COS cells readily (Table 1). The behavior was different in response to COS cells expressing Sema6A. Both DRG (Figure 6a) and sympathetic axons (Figure 6c) avoided Sema6A-positive cells. The effect was stronger for sympathetic neurons, where avoidance was found for 68% of the axons compared to 53% of the DRG axons (Table 1). Axons of motoneurons did not show a reaction to either Sema6A or Axonin-1 that differed from the behavior on EGFP-expressing cells (Figure 6b). Interestingly, we found that significantly more sympathetic axons reacted with attraction to Axonin-1 than to control COS cells expressing EGFP (Figure 6c). In conclusion, axons of DRG neurons were repelled by Sema6A, whereas motor axons did not react at all to Sema6A.

The effect of Sema6A in PNS/CNS border control is caused by a defect in BCC clustering

To gain insight into the mechanism of Sema6A function as a gate keeper, we analyzed the formation of BCC clusters in the absence of Sema6A from migrating neural crest cells (Figure 7). BCC clusters were reduced in size or missing altogether in the absence of Sema6A and their localization along the anteroposterior and the dorsoventral axes of the spinal cord was perturbed. The aberrant arrangement of BCC clusters was detectable both dorsally at the DREZ (Figure 7c) and ventrally at the VMEP (Figure 7f). At the dorsal root entry site 1E8-positive cells were no longer found in regular, dense clusters, as seen in control-treated embryos (Figure 7b). Many axons were not in close contact with BCCs in the absence of Sema6A (Figure

Table 1: DRG and sympathetic axons avoid Sema6A-expressing COS cells

	Sema6A			Axonin-1			EGFP		
	Avoid (%)	Stay (%)	Cross (%)	Avoid (%)	Stay (%)	Cross (%)	Avoid (%)	Stay (%)	Cross (%)
DRG	53.3 ± 4.7	5.9 ± 1.6	40.8 ± 4.7	15.5 ± 2.5	18.3 ± 3.0	66.1 ± 4.2	15.1 ± 2.2	9.0 ± 1.2	75.9 ± 1.7
MN	17.6 ± 4.6	9.5 ± 2.1	72.9 ± 4.0	14.6 ± 1.3	14.9 ± 2.1	70.5 ± 3.0	18.5 ± 3.6	10.2 ± 2.2	71.3 ± 1.4
SG	68.0 ± 2.6	3.1 ± 1.1	28.9 ± 3.4	2.9 ± 0.4	31.7 ± 1.1	65.4 ± 1.2	14.5 ± 1.7	14.9 ± 1.4	70.6 ± 3.1

COS cells transfected with Sema6A, Axonin-1, or EGFP were used as substrate for DRG, motor, and sympathetic neurons. For each condition at least 200 axons from 2 (sympathetic) or 3 (sensory and motoneurons) independent experiments were counted. Values represent mean ± standard error of the mean. MN, motoneuron; SG sympathetic neuron.

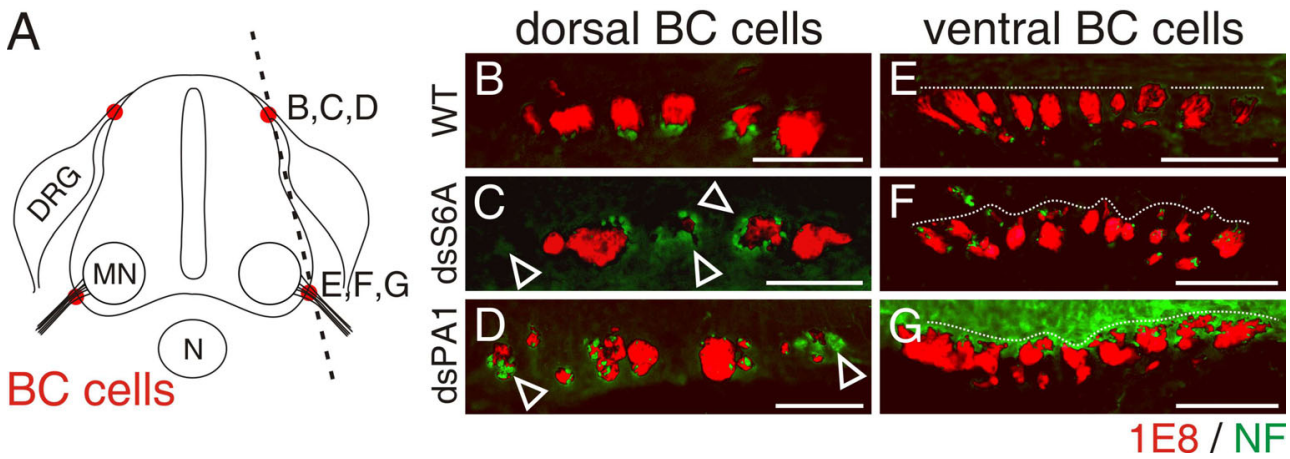


Figure 7

Clustering of BCCs is perturbed in the absence of Sema6A and PlexinA1. **(a-g)** Longitudinal sections of HH25 spinal cords (as indicated by the dashed line in (a)) were stained with 1E8 (red) and anti-neurofilament antibodies (green) to analyze dorsal (b-d) and ventral (e-g) BCCs from untreated embryos (b, e) or embryos treated with dsRNA derived from SEMA6A (c, f) and PLEXINA1 (d, g), respectively. Dorsal BCC clusters in control embryos (b) were relatively homogenous in size, closely aligned with the roots, and regularly spaced. In contrast, in the absence of either Sema6A (c) or PlexinA1 (d), the size of BCC clusters was very variable and their arrangement was highly disorganized. Many axons were not in contact with BCCs at all or only with individual cells or microclusters (open arrowheads in (c, d)). Ventral BCC clusters were smaller than their dorsal counterparts even in control embryos (e). Therefore, the effect of Sema6A (f) or PlexinA1 (g) perturbation on cluster size was less obvious. However, the absence of Sema6A and PlexinA1 clearly disrupted the alignment of ventral BCC clusters (compare dashed lines in (e) with (f, g)). The color of the axons stained with anti-neurofilament antibodies and visualized with an Alexa350-coupled secondary antibody was changed to green using Adobe Photoshop CS2 to get better contrast. EGFP used to select the appropriate sections is not shown. MN, motoneurons; N, notochord. Bar 100 μm.

7c). At the VMEP BCC clusters were smaller than their dorsal counterparts (compare Figure 7e and 7b). The downregulation of Sema6A in ventral BCCs resulted in their aberrant clustering along both the anteroposterior and the dorsoventral axes.

Similarly, downregulation of PlexinA1 in sensory (Figure 7d) and motoneurons (Figure 7g) resulted in the same aberrant arrangement of BCC clusters as seen after interference with Sema6A expression. As none of the PlexinAs was expressed in BCCs [20] and no homophilic interaction of Sema6A was found *in vitro* (data not shown), we concluded that Sema6A on BCCs was necessary to recognize a stop signal on sensory and motor axons. This signal was likely provided by PlexinA1, as axons were not decorated with 1E8-positive BCCs in the absence of it (Figure 7d). In support of this hypothesis, we found binding of AP-tagged Sema6A to both commissural and motor axons but not to BCCs, in accordance with the expectation that Sema6A would bind only to PlexinA-expressing cells and not to Sema6A-expressing cells (Figure 8a).

As an alternative approach to block the interaction between PlexinAs on motor axons and Sema6A on BCCs, we expressed the ectodomain or full-length Sema6A in motone1475-2875-6-162-6urons, where normally no Sema6A is found in chick (except for a transient expression at HH26; Figure 1m). Providing Sema6A on motor axons would prevent PlexinA1 from interacting with Sema6A on BCCs because it would compete with BCC-derived Sema6A. BCC clusters would thus fail to form properly due to the absence of the stop signal (Figure 8e) and motoneurons would stream out of the spinal cord at the VMEP just as found after either Sema6A downregulation in BCCs or PlexinA1 downregulation in motoneurons. This is indeed what we observed (Figure 8c).

In summary, our results support the hypothesis that Sema6A on BCCs interacts with PlexinA1 on motor axons to recognize the VMEP, where BCCs aggregate and cluster to form a barrier for motor neurons but not motor axons. If the BCC clusters fail to form properly, they cannot fulfill this barrier function and motoneurons stream out of the spinal cord along the ventral roots.

Discussion

Entry and exit sites of the CNS are well controlled transition areas that are permissive for axons but not for cell bodies during development due to the presence of the BCCs. The boundary cap is a transient structure that disappears at postnatal day 6 in the rat [49]. In chicken, BCC clusters labeled by KROX20 or SEMA6A disappear

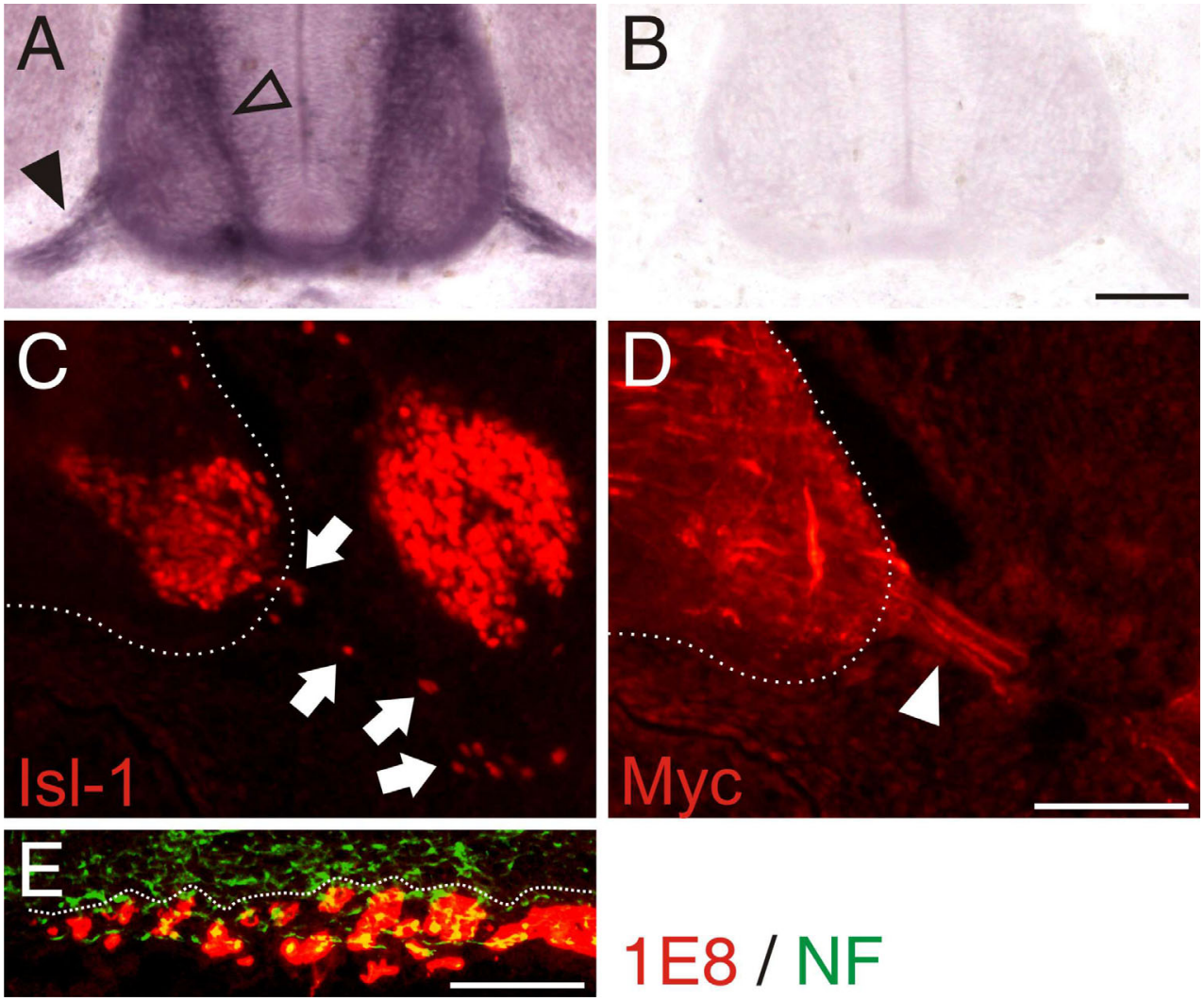


Figure 8

Ectopic expression of the Sema6A ectodomain or full-length Sema6A in motoneurons competes with BCC-derived Sema6A binding to motoneurons. The AP-tagged ectodomain of Sema6A binds to axons expressing PlexinAs. **(a)** Both commissural axons (open arrowhead) and motor axons (arrowhead) express PlexinAs [20] and bind the Sema6A ectodomain. **(b)** No binding of the AP-tag alone was detectable. **(c)** Ectopic expression of both the ectodomain of Sema6A (not shown) and the full-length myc-tagged form resulted in motoneurons streaming out of the spinal cord along the ventral roots (arrows). **(d)** Staining of the myc tag demonstrates expression of Sema6A in motor axons (arrowhead), consistent with a competitive role of motor axon-derived Sema6A with BCC-derived Sema6A in the periphery. **(e)** As seen after downregulation of either Sema6A in BCCs (compare to Figure 7f) or PlexinA1 in motoneurons (compare to Figure 7g), ectopic expression of Sema6A resulted in the aberrant formation of BCC clusters. Bar: 100 μm.

between HH36 and HH40 (Figure 1). They are replaced by a non-permissive barrier at the CNS/PNS interface consisting of astrocytes and Schwann cells [36,49]. BCCs originate from a late-migrating population of neural crest cells [38]. So far, they had been identified only after clustering by their expression of KROX20 and Cadherin-7. A time course of SEMA6A expression analyzed in transverse sections from the lumbosacral region of the embryonic chicken spinal cord suggests that BCCs express SEMA6A while they still migrate toward and cluster at the entry and exit sites of the spinal cord (Figure 1). The confined expression of SEMA6A in boundary cap cells together with the striking observation by Vermeren and colleagues [37] that ablation of BCC clusters resulted in the emigration of motoneurons from the ventral spinal cord into the periphery motivated us to test for a role of Sema6A in BCCs as a gate keeper between the CNS and the PNS. Indeed, we found that knock-down of Sema6A resulted in the same phenotype as ablation of the boundary cap (compare Figure 2 to [37]). In the absence of Sema6A

from BCCs, motoneurons left the spinal cord along the ventral roots. This effect was specific for loss of Sema6A function. Downregulation of other class 6 semaphorins did not enhance the number of motoneurons found outside the spinal cord compared to control-treated embryos. The fact that we could detect a phenotype of Sema6D loss of function for the dorsal root entry site but not for the ventral motor exit point further confirms the specificity of our approach. Downregulation of a target gene with long dsRNA was specific and efficient, as shown previously [48,50]. The specificity of downregulation was also corroborated by the use of dsRNA derived from a second non-overlapping fragment of cDNA from the 3' end of SEMA6A (data not shown).

Motoneurons leaving the spinal cord were only found after downregulation of Sema6A, while the effect at the dorsal root entry site was also seen after perturbation of Sema6D function, despite the fact that SEMA6D was expressed in ventral and dorsal BCCs. Similarly, downregulation of PlexinA1 had an effect at both the VMEP and the DREZ; loss of PlexinA4 function had an effect only dorsally. The phenotype observed after perturbation of PlexinA1 and PlexinA4 differed from loss of Sema6A/6D function, consistent with a role of class A plexins as receptors for secreted class 3 semaphorins. In the absence of PlexinA1, DRGs were misplaced along the rostrocaudal axis and they were not clearly segregated from each other (Figure 5). These observations are in agreement with studies reporting a role of plexin/neuropilin complexes in the restriction of neural crest migration to the anterior somite [51,52]. Restricted migration through the anterior somite was shown to be essential for the segmental organization of the PNS [53-55]. Thus, in the absence of PlexinA1, not only did dorsal roots fail to segregate properly, as seen after loss of Sema6A function, but the arrangement of the DRGs was also perturbed.

Based on our results, we propose a model where Sema6A in BCCs is required for them to home in on the entry and exit sites of the spinal cord, where they form the boundary cap (Figure 9). PlexinA1 on axons provides the stop signal that is recognized by Sema6A on migrating boundary cap cells. Because we were unable to detect a homophilic Sema6A interaction and none of the PlexinAs is expressed in BCCs, Sema6A is unlikely to be responsible for BCC clustering directly, that is, by mediating cell-cell contact between BCCs. Cadherin-7 is a good candidate for the adhesion molecule that might be responsible for the formation of tight cell-cell contacts between BCCs. Cadherin-7 is expressed strongly when BCCs have reached the aggregation site but not while they are still migrating, and it was shown to bind homophilically [47,56].

According to our model, Sema6A would act as a receptor when expressed in BCCs and recognize PlexinA1 as a ligand. A receptor role for Sema6A has been suggested previously in the brain, where Sema6A was shown to be required for the appropriate targeting of thalamocortical axons [27]. Similarly, a receptor function for Sema6D in

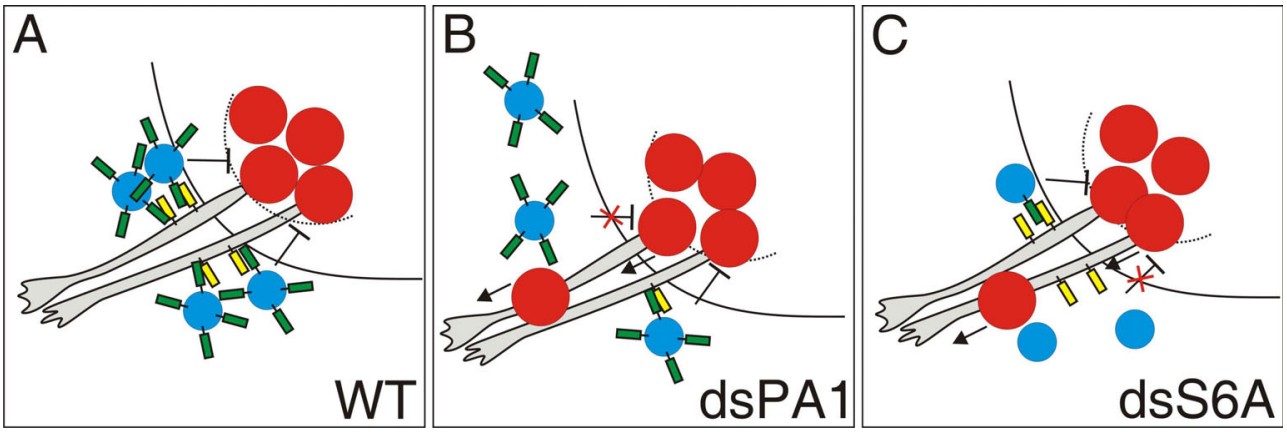


Figure 9
Sema6A acts as a gate keeper at the VMEP by triggering the formation of BCC clusters. Our results support a model that suggests a role for Sema6A in BCC cluster initiation. **(a)** Motor axons leaving the ventral spinal cord express PlexinA1 on their surface (yellow rectangle). Boundary cap cells (blue circles) express Sema6A (green rectangles), which recognizes PlexinA1 on motor axons, resulting in the accumulation of BCCs and, subsequently, in their clustering. By an unknown mechanism the BCC cluster prevents motor neurons (red circles) but not motor axons from translocating into the periphery. **(b, c)** Consistent with this model, the absence of PlexinA1 from motor axons would remove the stop signal (b) and the absence of Sema6A from BCCs would remove the receptor for the stop signal (c). In both cases, BCC clusters would fail to form properly and motoneurons would not be confined to the ventral spinal cord but migrate into the periphery along the ventral roots. The behavior of sensory axons at the dorsal BCC clusters is more complex and cannot be fully explained by this model.

neural crest cell migration in heart development was described [23,24]. A receptor function for class 6 semaphorins is also consistent with structural features [26].

Our model is supported by the aberrant clustering of BCCs in the absence of either Sema6A from BCCs or the absence of PlexinA1 from motoneurons (Figure 7). In the absence of Sema6A, BCCs fail to recognize the exit site marked by the first motor axons extending into the periphery, the boundary cap fails to form correctly and, as a consequence, motoneurons are no longer confined to the ventral spinal cord and migrate into the periphery following their axons (Figure 9). The same effect is achieved when PlexinA1 is downregulated in motoneurons. In this case motor axons are unable to provide a stop signal for migrating BCCs. Similarly, the PlexinA1 stop signal can be masked by expression of soluble Sema6A ectodomain or full-length Sema6A in motoneurons. In both cases motor axon-derived Sema6A would compete with Sema6A on the surface of BCCs and result in the aberrant formation of BCC clusters.

In addition to its function as a stop signal for Sema6A-expressing BCCs, PlexinA1 serves as a co-receptor together with neuropilins for class 3 semaphorins. Sema3A was postulated to act as a surround repellent and, thus, to polarize growth of sensory axons during initial stages of development [57]. Later, class 3 semaphorins were shown to interfere with motor and sensory axon pathfinding [2,58-64]. Their effects were mediated by binding to either Neuropilin-1 or Neuropilin-2 associated with one of the class A plexins as the signal transducing part of the receptor.

Chicken embryos express only three PlexinAs, as the gene encoding PlexinA3 is missing from the chicken genome [20]. Similarly, chickens express only three class 6 semaphorins; an ortholog of Sema6C is not found. Therefore, a direct comparison of PlexinA/Sema6 interactions between mouse/human and chicken proteins is not possible. This may explain why, so far, a direct interaction between Sema6A and PlexinA1 has not been demonstrated [25]. The repulsive activity of Sema6A was found to be mediated by PlexinA4 [22,65]. In our *in vivo* assays PlexinA4 had an effect only at the dorsal root entry but not at the ventral motor axon exit site. In our *in vitro* assay, sensory but not motor axons were repelled by Sema6A, despite the fact that all PlexinAs were expressed by sensory and motor neurons [20]. Sema6D was expressed in both dorsal and ventral BCCs but had an effect only at the dorsal root entry zone.

Future experiments will have to elucidate the difference between Sema6A and Sema6D in BCCs and, thus, their different roles in gate keeping between the PNS and the CNS. Obviously, the mechanism differs between the ventral and the dorsal transition zone. Motor axons were not repelled by Sema6A but sensory axons were (Figure 6 and Table 1). The reason for this discrepancy is unknown.

Conclusion

Sema6A expression by BCCs acts as a gate keeper between the PNS and the CNS by organizing the segregation of dorsal root entry and ventral motor axon exit sites. In both cases Sema6A on BCCs appears to act as a receptor recognizing the stop signal provided by PlexinA1 on axons. As a consequence, BCCs aggregate at the dorsal root entry site and the VMEP. BCCs then form clusters, possibly mediated by Cadherin-7, resulting in a tight barrier that prevents motor neurons from streaming out of the ventral spinal cord along the ventral roots. At the dorsal root entry site the BCCs segregate and organize dorsal roots. Consistent with these observations, Sema6A was found to be a repellent for sensory but not for motor axons.

Materials and methods

Cloning of the chicken SEMA6A cDNA

A 728 base-pair fragment of chicken SEMAPHORIN6A obtained in a screen for axon guidance cues [50] was used to screen a λ ZAP library prepared from E14 chicken brains [66]. Two fragments encoding the entire open reading frame (ORF) were ligated and cloned into pBluescript. For the preparation of *in situ* probes and dsRNA we used mainly a fragment spanning the 5' untranslated region and the first 300 base-pairs from the ORF. In addition, we verified the specificity of the phenotype using a fragment from the 3' untranslated region. The alignment of these fragments with SEMA6B and SEMA6D did not result in any significant similarity.

To obtain a soluble AP-tagged ectodomain of Sema6A, the sequence corresponding to the ectodomain of chicken Sema6A (amino acids 1–604) was amplified and inserted into the APtag-2 vector [67]. COS7 cells were transiently transfected with the Sema6A ectodomain-containing plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After transfection cells were washed with phosphate-buffered saline (PBS) and grown for 4 days in MEM and 1% fetal calf serum. The supernatant was collected and centrifuged as described in [68]. Binding of AP-tagged Sema6A to cryosections was carried out as described in [69]. Full-length Sema6A with a myc tag was expressed under the control of the β-actin promoter. The plasmid was injected at a concentration of 1 μg/μl into the central canal of the spinal cord of E2.5 embryos followed by electroporation of the ventral spinal cord. The embryos were sacrificed at E5 and analyzed.

Preparation of in situ *probes and dsRNA*

Probes for *in situ* hybridization and dsRNA were produced from expressed sequence tags (ESTs) obtained from Geneservice Ltd [70]. The ESTs used were: ChEST225N10 (SEMAPHORIN6D), ChEST53D13 and 666O16 (PLEXINA1), ChEST128L21 and 297D11 (PLEXINA2), ChEST1014M19 and 202O14 (PLEXINA4). For SEMAPHORIN6A the cDNA fragment mentioned above was used. For SEMA6B a cDNA fragment was cloned using RT-PCR because no ESTs were available. Total RNA was prepared from HH30 (stage 30 chicken embryos according to Hamburger and Hamilton [42]) spinal cords. Random and oligo dT-primed first-strand cDNAs were generated using Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). A 656 base-pair fragment for SEMA6B was amplified using the antisense primer 5'-CCCATGTCGTTCTTGCAC-3' and the sense primer 5'-ATCCAGCGCATCCTCAAG-3'. The resulting PCR fragments were cloned into the TOPO TA cloning vector (Invitrogen) using *Eco*RI restriction sites (Gemayel *et al.*, in preparation). We carefully compared and selected sequences to avoid overlapping stretches that could potentially interfere with RNAi specificity. In fact, off-target effects or unspecific knock-down of related family members were never detected in our approach with long dsRNA, most likely because the concentration of each small interfering RNA produced in a given cell by Dicer is extremely low, with a theoretical maximal concentration of about 1 nM or less [48,50].

In situ probes for the detection of KROX20 and SOX10 mRNA were derived from ESTs 738N7 and 477F10, respectively. Plasmid DNA was linearized using restriction enzymes *Not*I, *Eco*RI, *Xba*I, *Hin*dIII, or Asp718 (all from Roche, Basel, Switzerland) to prepare either digoxigenin-labeled *in situ* probes [20] or dsRNA [48] by *in vitro* transcription as described previously.

In ovo *RNAi*

In ovo RNAi was used to knock down genes of interest as described previously [48]. In brief, fertilized eggs were windowed on the second day of incubation to get access to the embryo. Embryos were staged according to Hamburger and Hamilton [42] at the time of injection. A solution containing the dsRNA (200–300 ng/μl) and a plasmid encoding EGFP under the control of the β-actin promoter (50 ng/μl) was injected into the central canal of the spinal cord of HH12-14 embryos to efficiently transfect neural crest cells and motoneurons [71]. The lumbosacral region of the spinal cord was electroporated with 5 pulses of 18 Volts and 50 ms length with a 1 s interpulse interval. Eggs were sealed and put back into the incubator until embryos reached the desired stage. Embryos were sacrificed at HH25 for the analysis of motoneurons and at HH25/26 for the analysis of dorsal roots.

Tissue preparation

For analysis of phenotypes, embryos were sacrificed, eviscerated and fixed in 4% paraformaldehyde in PBS for 60' to 120' depending on the age. Embryos were rinsed in PBS and subjected to cryoprotection or used directly for whole-mount staining as detailed below. For immunohistochemistry and *in situ* hybridization, the cryoprotected tissue was frozen in isopentane on dry ice and cut into 25 μm thick sections. In situ hybridization was carried out as detailed previously [20]. For immunohistochemistry, the staining protocol described earlier [72] was used. Antibodies were diluted in blocking buffer (10% fetal calf serum in PBS). For permeabilization of the tissue, sections were incubated for 1' in 0.1% Triton-X-100. The antibodies used were: monoclonal antibodies 1E8 recognizing P0, 40.2D6 recognizing Isl-1, and 9E10 recognizing the myc tag (all from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) Furthermore, we used rabbit anti-neurofilament (Millipore, Billerica, MA), and a FITC-labeled goat anti-GFP antibody (Rockland, Gilbertsville, PA). Secondary antibodies were: goat anti-mouse IgG Cy3 (Jackson ImmunoResearch Newmarket, Suffolk, UK), goat anti-rabbit Alexa350, and goat anti-rabbit Alexa488 (both Invitrogen/Molecular Probes, Carlsbad, CA).

Neurofilament staining of whole-mount embryos

For whole-mount staining, embryos were sacrificed at HH25/26, fixed as described above and transferred to 24-well plates. Tissue was permeabilized in 1% Triton/PBS for 1 h at room temperature, rinsed in PBS, and incubated in 20 mM lysine in 0.1 M sodium phosphate (pH 7.3) for another hour. After rinsing thoroughly in PBS, embryos were incubated in blocking buffer (10% fetal calf serum in PBS) for at least two hours before the anti-neurofilament antibody (RMO270 from Zymed/Invitrogen, Carlsbad, CA, diluted 1:1,500) was added for 48 h at 4°C. Incubation with the secondary antibody (goat anti-mouse IgG Cy3, 1:250) was for 12 h. EGFP was visualized with a FITC-labeled goat anti-GFP antibody. Embryos were rinsed thoroughly and dehydrated in a graded series of methanol before transfer to benzyl benzoate/benzyl alcohol (2:1).

Quantification of the phenotypes

Experimental embryos and control-treated embryos that were injected and electroporated with the plasmid encoding EGFP only were sacrificed at HH25/26. Tissue preparation, cutting and staining was as detailed above. From each embryo lumbosacral sections were analyzed by an observer who was blind to the treatment group. Sections were classified into groups containing either 0–1, or more than one Isl-1-positive cell along the root. All sections that contained EGFP and the ventral roots were analyzed and scored. The percentage of sections per embryo containing

more than one motoneuron outside the spinal cord was calculated.

For the analysis of the phenotype at the dorsal root entry site, embryos were sacrificed at HH25/26 and stained with RMO270 and goat anti-mouse IgG Cy3 as whole-mounts as detailed above. For the analysis of the segregation of DRGs and dorsal roots, a dissection microscope equipped with fluorescence optics (Olympus SZX12) was used. Single fibers crossing to the adjacent DRG or irregular spacing was considered a weak phenotype. When roots were formed by sensory axons emanating from two DRGs or when the DRGs were fused, the embryo was scored as having a severe phenotype. For statistical analysis, we used two-way ANOVA with Bonferroni correction. Values represent mean ± standard error of the mean.

In vitro *assay*

COS7 cells grown on 8-well LabTek slides were transfected with pcDNA3.1 vectors containing myc-tagged SEMA6A, myc-tagged AXONIN-1, or farnesylated EGFP (Invitrogen) as a control using Lipofectamine 2000 (Invitrogen). Sensory and sympathetic ganglia were dissected from HH26 or HH35 embryos. Motoneurons were obtained from the ventral halves of spinal cords dissected from HH26-28 embryos. Single-cell suspensions were obtained by digestion of ganglia and ventral spinal cord junks with trypsin followed by trituration. Per well, 25,000 DRG or sympathetic neurons or twice as many motoneurons were plated. DRG and sympathetic neurons were cultured in serum-free medium containing 20 ng/ml nerve growth factor (NGF) (see [73] for details). Motoneurons were cultured in MEM containing 5% fetal calf serum, N3, and 1 mM sodium pyruvate. Neurons were grown on transfected COS cells for one (DRG, sympathetic neurons) or two days (motoneurons) before fixation in 4% paraformaldehyde for 30 minutes at room temperature and staining with the 9E10 antibody (Developmental Studies Hybridoma Bank) to detect successfully transfected cells and rabbit anti-neurofilament to stain axons.

Cultures were analyzed and the behavior of axons encountering a transfected COS cell was classified as avoidance if an axon stopped or turned away from a transfected cell, or as attraction if an axon failed to leave the surface of a transfected cell. We chose Axonin-1 as a control protein because it was shown to promote axon outgrowth of sensory neurons [73,74]. In addition, Axonin-1 was shown to be required for pathfinding of nociceptive afferents [75] and axons of dorsolateral commissural neurons [48,76] but not for extension of commissural axons [77].

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

OM, ED, IA, RS, and ES carried out experiments and analyzed the data. OM prepared the figures. ES conceived the study and wrote the manuscript.

Additional material

Additional file 1

Downregulation of the targeted PlexinA was specific. For each PlexinA we used two different cDNA fragments to produce long dsRNAs for gene silencing (see Materials and methods for details). As shown qualitatively by in situ *hybridization (a-c, e-g, i-k) and quantitatively by analyzing intensity levels for the three PlexinAs with ImageJ 1.38× (m) downregulation of the targeted PlexinA was specific. Downregulation of PLEXINA1 (PA1; a-d) resulted in a reduction of PA1 expression in motoneurons (arrowhead in (a)). Pattern and expression levels of PA2 (b) and PA4 (c) were not changed. EGFP expression from a co-injected plasmid indicates the electroporated half of the spinal cord (d). Similarly, silencing of PA2 (e-h) resulted in changes of the PA2 expression pattern in motoneurons (arrow in (f)) on the electroporated side (see (h)) but the expression of PA1 (e) and PA4 (g) were not altered. Targeting PA4, which is very weak at HH25, further reduced expression of PA4 in motoneurons (arrowhead in (k)) on the electroporated side (l) but had no effect on the expression of PA1 (i) and PA2 (j). The quantification of the signal intensity is shown in (m). Similar levels were obtained for all PlexinAs. Three or four different embryos were analyzed per condition using at least ten sections. Specific downregulation was 24.9 ± 5.7% for PA1, 26.8 ± 5.5% for PA2, and 25.9 ± 2.1% for PA4. Values are shown ± standard deviation.* $P < 0.0001$ *indicated by three asterisks. Note that the section shown in (f) was taken from the thoracic level. AS, antisense probe.*

Click here for file

[http://www.biomedcentral.com/content/supplementary/1749-8104-2-28-S1.jpeg]

Acknowledgements

We thank Maja Hess for excellent technical assistance, members of the lab for suggestions and reading the manuscript. This work was supported by the Swiss National Science Foundation and the NCCR Brain Plasticity and Repair.

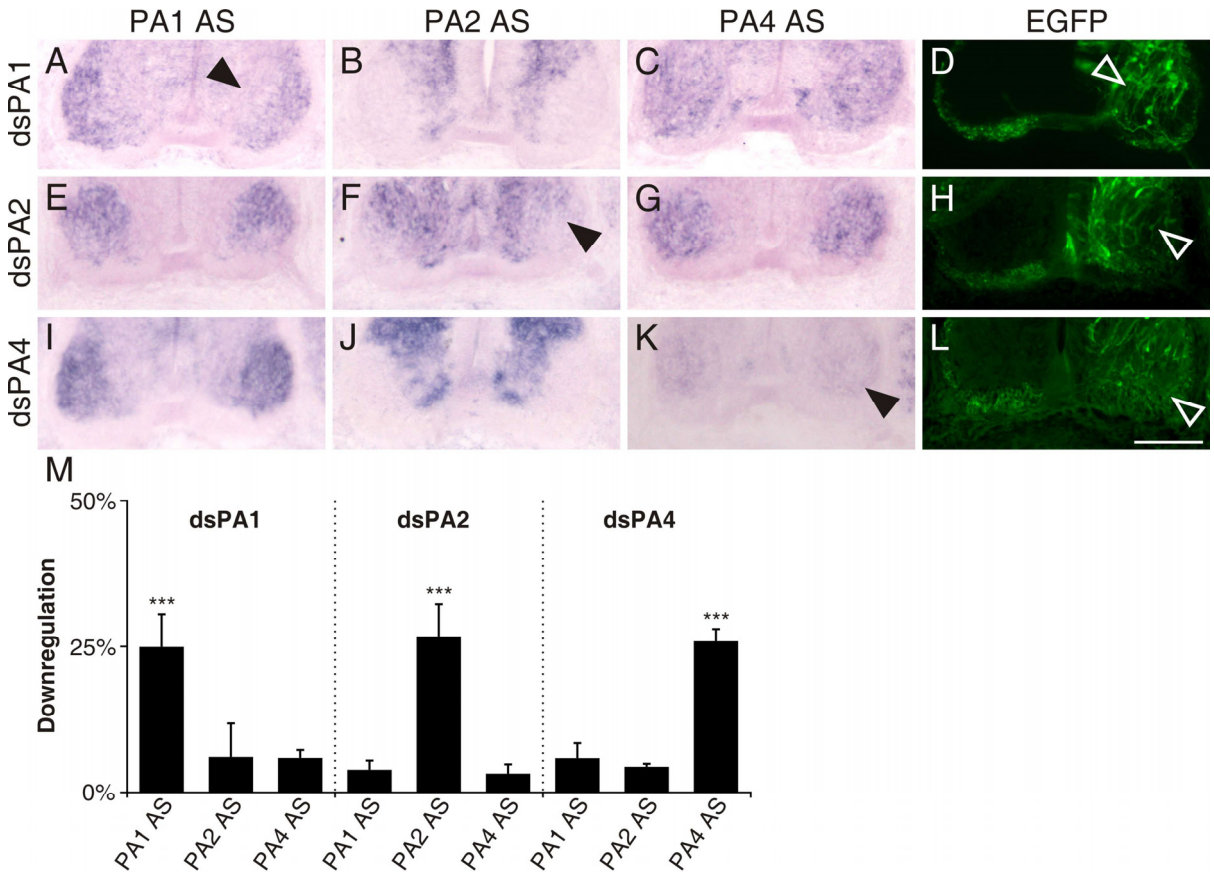
References

1. Dickson BJ: **Molecular mechanisms of axon guidance.** *Science* 2002, **298:**1959-1964.
2. Huber AB, Kolodkin AL, Ginty DD, Cloutier JF: **Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance.** *Annu Rev Neurosci* 2003, **26:**509-563.
3. Rougon G, Hobert O: **New insights into the diversity and function of neuronal immunoglobulin superfamily molecules.** ***Annu Rev Neurosci*** 2003, **26:**207-238.
4. Egea J, Klein R: **Bidirectional Eph-ephrin signaling during axon guidance.** *Trends Cell Biol* 2007, **17:**230-238.
5. Baker KA, Moore SW, Jarjour AA, Kennedy TE: **When a diffusible axon guidance cue stops diffusing: roles for netrins in adhesion and morphogenesis.** *Curr Opin Neurobiol* 2006, **16:**529-534.
6. Cirulli V, Yebra M: **Netrins: beyond the brain.** *Nat Rev Mol Cell Biol* 2007, **8:**296-306.
7. Yazdani U, Terman JR: **The semaphorins.** *Genome Biol* 2006, **7:**211.

8. Tran TS, Kolodkin AL, Bharadwaj R: **Semaphorin regulation of cellular morphology.** *Annu Rev Cell Dev Biol* 2007, **23:**263-292.
9. Negishi M, Oinuma I, Katoh H: **Plexins: axon guidance and signal transduction.** *Cell Mol Life Sci* 2005, **62:**1363-1371.
10. Fujisawa H: **Discovery of semaphorin receptors, neuropilin and plexin, and their functions in neural development.** *J Neurobiol* 2004, **59:**24-33.
11. Bovolenta P: **Morphogen signaling at the vertebrate growth cone: A few cases or a general strategy?** *J Neurobiol* 2005, **64:**405-416.
12. Stoeckli ET: **Longitudinal axon guidance.** *Curr Opin Neurobiol* 2006, **16:**35-39.
13. Zou Y, Lyuksyutova AI: **Morphogens as conserved axon guidance cues.** *Curr Opin Neurobiol* 2007, **17:**22-28.
14. de Wit J, Verhaagen J: **Role of semaphorins in the adult nervous system.** *Prog Neurobiol* 2003, **71:**249-267.
15. Fiore R, Puschel AW: **The function of semaphorins during nervous system development.** *Front Biosci* 2003, **8:**s484-499.
16. Gu C, Yoshida Y, Livet J, Reimert DV, Mann F, Merte J, Henderson CE, Jessell TM, Kolodkin AL, Ginty DD: **Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins.** *Science* 2005, **307:**265-268.
17. Worzfeld T, Puschel AW, Offermanns S, Kuner R: **Plexin-B family members demonstrate non-redundant expression patterns in the developing mouse nervous system: an anatomical basis for morphogenetic effects of Sema4D during development.** *Eur J Neurosci* 2004, **19:**2622-2632.
18. Cohen S, Funkelstein L, Livet J, Rougon G, Henderson CE, Castellani V, Mann F: **A semaphorin code defines subpopulations of spinal motor neurons during mouse development.** *Eur J Neurosci* 2005, **21:**1767-1776.
19. Perala NM, Immonen T, Sariola H: **The expression of plexins during mouse embryogenesis.** *Gene Expr Patterns* 2005, **5:**355-362.
20. Mauti O, Sadhu R, Gemayel J, Gesemann M, Stoeckli ET: **Expression patterns of plexins and neuropilins are consistent with cooperative and separate functions during neural development.** ***BMC Dev Biol*** 2006, **6:**32.
21. Kruger RP, Aurandt J, Guan KL: **Semaphorins command cells to move.** *Nat Rev Mol Cell Biol* 2005, **6:**789-800.
22. Suto F, Ito K, Uemura M, Shimizu M, Shinkawa Y, Sanbo M, Shinoda T, Tsuboi M, Takashima S, Yagi T, *et al.*: **Plexin-a4 mediates axon-repulsive activities of both secreted and transmembrane semaphorins and plays roles in nerve fiber guidance.** *J Neurosci* 2005, **25:**3628-3637.
23. Toyofuku T, Zhang H, Kumanogoh A, Takegahara N, Suto F, Kamei J, Aoki K, Yabuki M, Hori M, Fujisawa H, Kikutani H: **Dual roles of Sema6D in cardiac morphogenesis through region-specific association of its receptor, Plexin-A1, with off-track and vascular endothelial growth factor receptor type 2.** *Genes Dev* 2004, **18:**435-447.
24. Toyofuku T, Zhang H, Kumanogoh A, Takegahara N, Yabuki M, Harada K, Hori M, Kikutani H: **Guidance of myocardial patterning in cardiac development by Sema6D reverse signalling.** ***Nat Cell Biol*** 2004, **6:**1204-1211.
25. Yoshida Y, Han B, Mendelsohn M, Jessell TM: **PlexinA1 signaling directs the segregation of proprioceptive sensory axons in the developing spinal cord.** *Neuron* 2006, **52:**775-788.
26. Klostermann A, Lutz B, Gertler F, Behl C: **The orthologous human and murine semaphorin 6A-1 proteins (SEMA6A-1/Sema6A-1) bind to the enabled/vasodilator-stimulated phosphoprotein-like protein (EVL) via a novel carboxyl-terminal zyxin-like domain.** *J Biol Chem* 2000, **275:**39647-39653.
27. Leighton PA, Mitchell KJ, Goodrich LV, Lu X, Pinson K, Scherz P, Skarnes WC, Tessier-Lavigne M: **Defining brain wiring patterns and mechanisms through gene trapping in mice.** *Nature* 2001, **410:**174-179.
28. Godenschwege TA, Hu H, Shan-Crofts X, Goodman CS, Murphey RK: **Bi-directional signaling by Semaphorin 1a during central synapse formation in *Drosophila*.** *Nat Neurosci* 2002, **5:**1294-1301.
29. Yu HH, Araj HH, Ralls SA, Kolodkin AL: **The transmembrane Semaphorin Sema I is required in *Drosophila* for embryonic motor and CNS axon guidance.** *Neuron* 1998, **20:**207-220.
30. Sweeney LB, Couto A, Chou YH, Berdnik D, Dickson BJ, Luo L, Komiyama T: **Temporal target restriction of olfactory receptor neurons by Semaphorin-1a/PlexinA-mediated axon-axon interactions.** *Neuron* 2007, **53:**185-200.
31. Wong JT, Wong ST, O'Connor TP: **Ectopic semaphorin-1a functions as an attractive guidance cue for developing peripheral neurons.** *Nat Neurosci* 1999, **2:**798-803.
32. Cafferty P, Yu L, Long H, Rao Y: **Semaphorin-1a functions as a guidance receptor in the *Drosophila* visual system.** *J Neurosci* 2006, **26:**3999-4003.
33. Kerjan G, Dolan J, Haumaitre C, Schneider-Maunoury S, Fujisawa H, Mitchell KJ, Chedotal A: **The transmembrane semaphorin Sema6A controls cerebellar granule cell migration.** *Nat Neurosci* 2005, **8:**1516-1524.
34. Xu XM, Fisher DA, Zhou L, White FA, Ng S, Snider WD, Luo Y: **The transmembrane protein semaphorin 6A repels embryonic sympathetic axons.** *J Neurosci* 2000, **20:**2638-2648.
35. Burgaya F, Fontana X, Martinez A, Montolio M, Mingorance A, Simo S, del Rio JA, Soriano E: **Semaphorin 6C leads to GSK-3-dependent growth cone collapse and redistributes after entorhinohippocampal axotomy.** *Mol Cell Neurosci* 2006, **33:**321-334.
36. Golding JP, Cohen J: **Border controls at the mammalian spinal cord: late-surviving neural crest boundary cap cells at dorsal root entry sites may regulate sensory afferent ingrowth and entry zone morphogenesis.** *Mol Cell Neurosci* 1997, **9:**381-396.
37. Vermeren M, Maro GS, Bron R, McGonnell IM, Charnay P, Topilko P, Cohen J: **Integrity of developing spinal motor columns is regulated by neural crest derivatives at motor exit points.** *Neuron* 2003, **37:**403-415.
38. Niederlander C, Lumsden A: **Late emigrating neural crest cells migrate specifically to the exit points of cranial branchiomotor nerves.** *Development* 1996, **122:**2367-2374.
39. Maro GS, Vermeren M, Voiculescu O, Melton L, Cohen J, Charnay P, Topilko P: **Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS.** *Nat Neurosci* 2004, **7:**930-938.
40. Hjerling-Leffler J, Marmigere F, Heglind M, Cederberg A, Koltzenburg M, Enerback S, Ernfors P: **The boundary cap: a source of neural crest stem cells that generate multiple sensory neuron subtypes.** *Development* 2005, **132:**2623-2632.
41. Marmigere F, Ernfors P: **Specification and connectivity of neuronal subtypes in the sensory lineage.** *Nat Rev Neurosci* 2007, **8:**114-127.
42. Hamburger V, Hamilton HL: **A series of normal stages in the development of the chick embryo.** *J Morphol* 1951, **88:**49-92.
43. Wilkinson DG, Bhatt S, Chavrier P, Bravo R, Charnay P: **Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse.** *Nature* 1989, **337:**461-464.
44. Bhattacharyya A, Brackenbury R, Ratner N: **Axons arrest the migration of Schwann cell precursors.** *Development* 1994, **120:**1411-1420.
45. Cheng Y, Cheung M, Abu-Elmagd MM, Orme A, Scotting PJ: **Chick sox10, a transcription factor expressed in both early neural crest cells and central nervous system.** *Brain Res Dev Brain Res* 2000, **121:**233-241.
46. Bhattacharyya A, Frank E, Ratner N, Brackenbury R: **P0 is an early marker of the Schwann cell lineage in chickens.** *Neuron* 1991, **7:**831-844.
47. Nakagawa S, Takeichi M: **Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins.** *Development* 1995, **121:**1321-1332.
48. Pekarik V, Bourikas D, Miglino N, Joset P, Preiswerk S, Stoeckli ET: **Screening for gene function in chicken embryo using RNAi and electroporation.** *Nat Biotechnol* 2003, **21:**93-96.
49. Golding J, Shewan D, Cohen J: **Maturation of the mammalian dorsal root entry zone – from entry to no entry.** *Trends Neurosci* 1997, **20:**303-308.
50. Bourikas D, Pekarik V, Baeriswyl T, Grunditz A, Sadhu R, Nardo M, Stoeckli ET: **Sonic hedgehog guides commissural axons along the longitudinal axis of the spinal cord.** *Nat Neurosci* 2005, **8:**297-304.
51. Eickholt BJ, Mackenzie SL, Graham A, Walsh FS, Doherty P: **Evidence for collapsin-1 functioning in the control of neural crest migration in both trunk and hindbrain regions.** *Development* 1999, **126:**2181-2189.
52. Gammill LS, Gonzalez C, Gu C, Bronner-Fraser M: **Guidance of trunk neural crest migration requires neuropilin 2/semaphorin 3F signaling.** *Development* 2006, **133:**99-106.

53. Krull CE: **Segmental organization of neural crest migration.** ***Mech Dev*** 2001, **105:**37-45.
54. Kuan CY, Tannahill D, Cook GM, Keynes RJ: **Somite polarity and segmental patterning of the peripheral nervous system.** ***Mech Dev*** 2004, **121:**1055-1068.
55. Kasemeier-Kulesa JC, Kulesa PM, Lefcort F: **Imaging neural crest cell dynamics during formation of dorsal root ganglia and sympathetic ganglia.** *Development* 2005, **132:**235-245.
56. Nakagawa S, Takeichi M: **Neural crest emigration from the neural tube depends on regulated cadherin expression.** *Development* 1998, **125:**2963-2971.
57. Masuda T, Shiga T: **Chemorepulsion and cell adhesion molecules in patterning initial trajectories of sensory axons.** *Neurosci Res* 2005, **51:**337-347.
58. Chen H, Bagri A, Zupicich JA, Zou Y, Stoeckli E, Pleasure SJ, Lowenstein DH, Skarnes WC, Chedotal A, Tessier-Lavigne M: **Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections.** *Neuron* 2000, **25:**43-56.
59. Giger RJ, Cloutier JF, Sahay A, Prinjha RK, Levengood DV, Moore SE, Pickering S, Simmons D, Rastan S, Walsh FS, *et al.*: **Neuropilin-2 is required *in vivo* for selective axon guidance responses to secreted semaphorins.** *Neuron* 2000, **25:**29-41.
60. Kitsukawa T, Shimizu M, Sanbo M, Hirata T, Taniguchi M, Bekku Y, Yagi T, Fujisawa H: **Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice.** *Neuron* 1997, **19:**995-1005.
61. Taniguchi M, Yuasa S, Fujisawa H, Naruse I, Saga S, Mishina M, Yagi T: **Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection.** *Neuron* 1997, **19:**519-530.
62. Varela-Echavarria A, Tucker A, Puschel AW, Guthrie S: **Motor axon subpopulations respond differentially to the chemorepellents netrin-1 and semaphorin D.** *Neuron* 1997, **18:**193-207.
63. Zou Y, Stoeckli E, Chen H, Tessier-Lavigne M: **Squeezing axons out of the gray matter: a role for slit and semaphorin proteins from midline and ventral spinal cord.** *Cell* 2000, **102:**363-375.
64. Yaron A, Huang PH, Cheng HJ, Tessier-Lavigne M: **Differential requirement for Plexin-A3 and -A4 in mediating responses of sensory and sympathetic neurons to distinct class 3 Semaphorins.** *Neuron* 2005, **45:**513-523.
65. Suto F, Tsuboi M, Kamiya H, Mizuno H, Kiyama Y, Komai S, Shimizu M, Sanbo M, Yagi T, Hiromi Y, Chédotal A, *et al.*: **Interactions between Plexin-A2, Plexin-A4, and Semaphorin 6A control lamina-restricted projection of hippocampal mossy fibers.** ***Neuron*** 2007, **53:**535-547.
66. Zuellig RA, Rader C, Schroeder A, Kalousek MB, Von Bohlen und Halbach F, Osterwalder T, Inan C, Stoeckli ET, Affolter HU, Fritz A, *et al.*: **The axonally secreted cell adhesion molecule, axonin-1. Primary structure, immunoglobulin-like and fibronectin-type-III-like domains and glycosyl-phosphatidylinositol anchorage.** *Eur J Biochem* 1992, **204:**453-463.
67. Flanagan JG, Cheng HJ, Feldheim DA, Hattori M, Lu Q, Vanderhaeghen P: **Alkaline phosphatase fusions of ligands or receptors as *in situ* probes for staining of cells, tissues, and embryos.** *Methods Enzymol* 2000, **327:**19-35.
68. Flanagan JG, Leder P: **The kit ligand: a cell surface molecule altered in steel mutant fibroblasts.** *Cell* 1990, **63:**185-194.
69. Brennan C, Fabes J: **Alkaline phosphatase fusion proteins as affinity probes for protein localization studies.** *Sci STKE* 2003, **2003:**PL2.
70. **Geneservice Ltd** [http://www.geneservice.co.uk/]
71. Bourikas D, Baeriswyl T, Sadhu R, Stoeckli ET: ***In ovo* RNAi opens new possibilities for functional genomics in vertebrates.** In *RNA Interference Technology – From Basic Research to Drug Development* Edited by: Appasani K. Cambridge UK: Cambridge University Press; 2005:220-232.
72. Perrin FE, Stoeckli ET: **Use of lipophilic dyes in studies of axonal pathfinding *in vivo*.** *Microsc Res Tech* 2000, **48:**25-31.
73. Stoeckli ET, Kuhn TB, Duc CO, Ruegg MA, Sonderegger P: **The axonally secreted protein axonin-1 is a potent substratum for neurite growth.** *J Cell Biol* 1991, **112:**449-455.
74. Stoeckli ET, Ziegler U, Bleiker AJ, Groscurth P, Sonderegger P: **Clustering and functional cooperation of Ng-CAM and axonin-1 in the substratum-contact area of growth cones.** *Dev Biol* 1996, **177:**15-29.
75. Perrin FE, Rathjen FG, Stoeckli ET: **Distinct subpopulations of sensory afferents require F11 or axonin-1 for growth to their target layers within the spinal cord of the chick.** *Neuron* 2001, **30:**707-723.
76. Stoeckli ET, Landmesser LT: **Axonin-1, Nr-CAM, and Ng-CAM play different roles in the *in vivo* guidance of chick commissural neurons.** *Neuron* 1995, **14:**1165-1179.
77. Fitzli D, Stoeckli ET, Kunz S, Siribour K, Rader C, Kunz B, Kozlov SV, Buchstaller A, Lane RP, Suter DM, *et al.*: **A direct interaction of axonin-1 with NgCAM-related cell adhesion molecule (NrCAM) results in guidance, but not growth of commissural axons.** *J Cell Biol* 2000, **149:**951-968.

Supplementary Figure 1



6. Additional Materials and Methods

Temporal Control of Gene Silencing by *in ovo* Electroporation

Thomas Baeriswyl*, Olivier Mauti*, and Esther T. Stoeckli
(*equal contribution)

Institute of Zoology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

Published in Methods in Molecular Biology, 2008, 442:231-244

16

Temporal Control of Gene Silencing by *in ovo* Electroporation

Thomas Baeriswyl*, Olivier Mauti*, and Esther T. Stoeckli

Summary

The analysis of gene function during embryonic development asks for tight temporal control of gene expression. Classic genetic tools do not allow for this, as the absence of a gene during the early stages of development will preclude its functional analysis during later stages. In contrast, RNAi technology allows one to achieve temporal control of gene silencing especially when used with oviparous animal models. In contrast to mammals, reptiles and birds are easily accessible during embryonic development. We have developed approaches to use RNAi for the analysis of gene function during nervous system development in the chicken embryo. Although the protocol given here describes a method for gene silencing in the developing spinal cord, it can easily be adapted to other parts of the developing nervous system. The combination of the easy accessibility of the chicken embryo and RNAi provides a unique opportunity for temporal and spatial control of gene silencing during development.

Key Words: *in ovo* RNAi; *in ovo* electroporation; long dsRNA; chicken embryo; development; nervous system.

1. Introduction

No matter whether you want to analyze the function of a number of candidate genes that you identified in a screen or whether you want to assess the function of your favorite gene, you may need an *in vivo* system that allows for the rapid

* These authors contributed equally.

From: *Methods in Molecular Biology, vol. 442: RNAi: Design and Application*
Edited by: S. Barik © Humana Press, Totowa, NJ

detection of a possible phenotype during development. The analysis of gene function during development requires tight temporal control of gene silencing. Classic genetic tools will only allow for an assessment of gene function during the initial phase of gene activity. Additional activities during later developmental stages will not be within reach, as the lack of gene function during the early stages will preclude the analysis of all subsequent stages. For this reason, specific gene silencing by RNA interference (RNAi) provides an exceptional tool for loss-of-function approaches during development in vertebrates. Until now, different RNAi strategies have been established for mouse, rat, and chicken embryos ***(1–4***; reviewed in ***5–7)***. However, due to the limited accessibility of mouse and rat embryos during development, RNAi in combination with *in utero* electroporation is very difficult and requires special equipment and expert training. Therefore, the use of mammals as a model organism for developmental studies is limited. In contrast to mammals, the chicken is easily accessible for *in vivo* manipulations during embryonic development. With the establishment of *in ovo* electroporation, as an efficient method for nucleic acid transfer, and *in ovo* RNAi, as a method for gene silencing, the chicken embryo has been turned into a unique model organism for the efficient functional characterization of genes involved in developmental processes ***(4,8–12***; reviewed in ***6)***. *In ovo* RNAi using long dsRNA, shRNA, or siRNA has been used for a variety of functional studies in different parts of the CNS but also in other embryonic tissues ***(4,13–17***; reviewed in ***5)***.

Here we provide a detailed protocol for the silencing of a candidate gene during early development of the spinal cord by *in ovo* RNAi. A particular advantage of *in ovo* RNAi is the fact that long dsRNA can be used for the induction of loss-of-function phenotypes. Unlike adult tissue or cell lines, embryos do not respond to long dsRNA with unspecific inhibition of protein synthesis and apoptosis ***(18,19)***. Therefore, there is no need for lengthy selection of an efficient siRNA or shRNA. Any cDNA fragment or expressed sequence tag (EST) can be used to produce dsRNA by *in vitro* transcription. Since the chicken genome was fully sequenced in 2004, it can be directly compared with the human, mouse, or rat genome, significantly facilitating the identification of orthologs ***(20)***. Therefore, *in ovo* RNAi offers the possibility to study candidate genes identified in other species using commercially available chicken ESTs for the synthesis of the dsRNA.

In the protocol reported here, long dsRNA is injected into the central canal of the developing spinal cord (**Fig. 1a**). Subsequently, the embryo is exposed to an electric field for efficient transfection of selected cell populations (**Fig. 1b**). Depending on the time point and the position of the electrodes, different neuronal populations within the spinal cord (**Figs. 1c, 1d**) but also of the peripheral nervous system can be targeted. Furthermore, this method allows for knockdown of several genes by injecting a mixture of different dsRNAs.

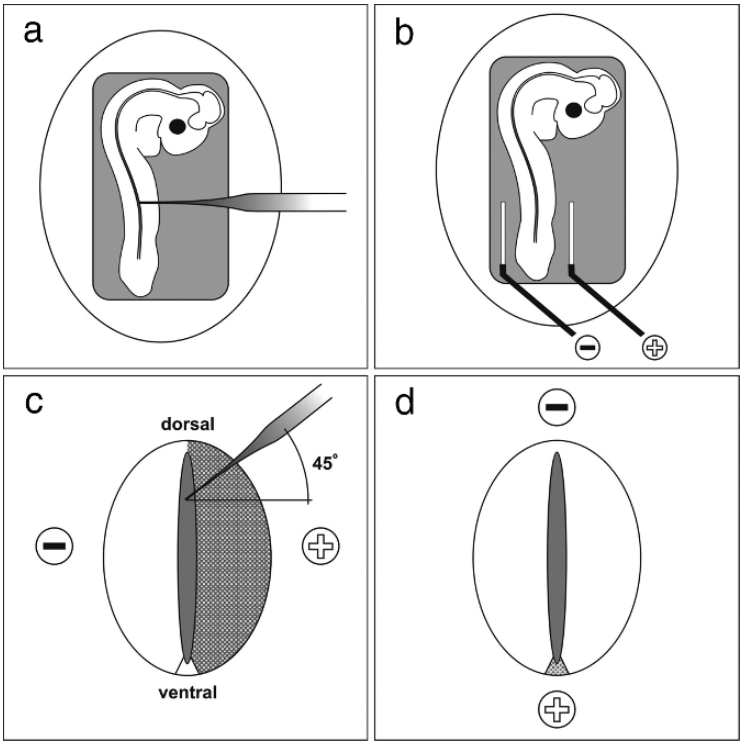


Fig. 1. *In ovo* electroporation. (a) The chicken embryo is made directly accessible through a window in the eggshell for the injection of nucleic acids into the central canal of the spinal cord. (b) The electroporation permeabilizes the cell membrane and therefore allows for the efficient uptake of RNA or DNA. Depending on the position of the electrodes with respect to the embryonic body axis, different tissues can be targeted: A parallel position of the electrode to the spinal cord results in a unilateral transfection [checkered area in (c)]. Within the applied electric field, the injected nucleic acids migrate toward the anode due to the negative charge of RNA and DNA. Therefore, the untransfected side of the spinal cord [left side in (c)] can be used as an internal control. The capillary should be kept at a 45° angle for injection. Placing the electrodes over the dorsal (cathode) and the ventral (anode) midline of the spinal cord results in efficient targeting of floor-plate cells [checkered area in (d)].

Thus, *in ovo* RNAi represents an efficient and inexpensive method to alter the expression of specific genes in a temporally and spatially controlled manner.

2. Materials

2.1. Preparation of dsRNA by in vitro Transcription

1. Heating block at 95 °C.
2. Equipment for gel electrophoresis.

3. *Bam*HI restriction endonuclease (10 U/µL; Roche, Basel, Switzerland).
4. *Sac*I restriction endonuclease (10 U/µL; Roche).
5. RNasin (40 U/µL; Promega, Madison, WI).
6. SP6 and T7 RNA polymerases (15 U/µL; Promega).
7. RNase-free DNaseI (10 U/µL; Roche).
8. 5X transcription buffer (Promega).
9. 100 m*M* rNTPs (25 m*M* of each rNTP; Roche).
10. 100 m*M* DTT (Promega).
11. 0.5 *M* EDTA (pH 8.0).
12. 7.5 *M* ammonium acetate.
13. Phenol–chloroform–isoamylalcohol (25:24:1 vol/vol/vol; pH 7.6–8.0).
14. Acidic phenol–chloroform–isoamylalcohol (25:24:1 vol/vol/vol; pH 4.0).
15. Chloroform–isoamylalcohol (24:1 vol/vol).
16. 100% ethanol.
17. DEPC-treated double-distilled water (ddH_2O) (1:1,000 vol/vol).
18. 70% ethanol in DEPC-treated ddH_2O.
19. Phosphate-buffered saline (PBS, DEPC-treated, 1:1,000 vol/vol): 137 m*M* NaCl, 2.7 m*M* KCl (*see* **Note 1**), 8 m*M* Na_2HPO_4, 1.5 m*M* NaH_2PO_4 (pH 7.4).
20. RNaseZAP (Sigma-Aldrich, St. Louis, MO).

2.2. Windowing the Eggs

1. Fertilized Hisex eggs were obtained from a local hatchery.
2. Preincubator: 38–39 °C, 45% humidity (JUPPITER 576 SETTER+HATCHER; F.I.E.M., Guanzate, Italy; *see* **Note 2**).
3. Incubator: 38–39 °C, 45% humidity (Heraeus/Kendro Model B12, Kendro Laboratory Products, Hanau, Germany; *see* **Note 2**).
4. Egg-Lume Candler (Brinsea Products Ltd., Sandford, UK).
5. Heating plate, 80 °C, to melt paraffin.
6. Soldering iron.
7. Scalpel for drilling holes.
8. 10-mL syringe with needle (Sterican 100, Ø 18G, B. Braun Melsungen AG, Melsungen, Germany).
9. Small scissors for cutting the eggshell (Fine Science Tools Inc., Foster City, CA).
10. Paraffin (Paraplast Tissue Embedding Medium, Oxford Labware, St. Louis, MO).
11. Coverslips, 24 × 24 mm (VWR International AG, Dietikon, Switzerland).
12. Kleenex.
13. Scotch tape.
14. 70% ethanol.

2.3. In ovo *Injection and Electroporation*

1. Spring scissors and forceps (Fine Science Tools Inc.).
2. Electroporator (Electro Square Porator ECM830, BTX Instrument Division, Harvard Apparatus Inc., Holliston, MA; *see* **Note 3**).

3. Platinum electrodes (4-mm length, 4 mm between anode and cathode; BTX Instrument Division, Harvard Apparatus Inc.; *see* **Note 3**).
4. Needle puller (PC-10, Narishige Co., Ltd., Tokyo, Japan).
5. Borosilicate glass capillaries (outer Ø/inner Ø: 1.2 mm/0.68 mm; World Precision Instruments, Sarasota, FL).
6. Polyethylene tubing (Ø 1.24 mm).
7. 0.2-µm filter (Sarstedt, Sevelen, Switzerland).
8. Reporter plasmid: EGFP under the control of the chicken β-actin promoter.
9. Trypan Blue solution 0.4% (Invitrogen, Carlsbad, CA).

3. Methods

3.1. Synthesis of Long dsRNA

A cDNA fragment of a candidate gene cloned into a standard plasmid containing SP6 (or T3) and T7 promoters flanking the insert can be used for the synthesis of long dsRNA by *in vitro* transcription. Here we synthesized dsRNA from a 678-bp cDNA fragment (1,620–2,298 bp) encoding Axonin-1 cloned in the pSP72 vector using SP6 and T7 promoters flanking the insert (*see* **Note 4**).

1. Linearize 10 µg of the plasmid with 20 U *Bam*HI and *Sac*I restriction endonuclease, respectively, for 1 h at 37 °C.
2. For *in vitro* transcription, mix 2 µg of the linearized plasmid with 0.8 µL of 100 m*M* rNTPs, 0.5 µL of RNasin, 2 µL of SP6 or T7 RNA polymerase, 4 µL of 5X transcription buffer, and 2 µL of 100 m*M* DTT, and add DEPC-treated ddH_2O to a total volume of 20 µL (*see* **Note 5**).
3. Incubate the *in vitro* transcription mixture for 2 h at 37 °C (T7 RNA polymerase) and 40 °C (SP6 RNA polymerase), respectively (*see* **Note 6**).
4. Remove the DNA template from the *in vitro* transcription mixture by adding 2 µL RNase-free DNaseI, and incubate at 37 °C for 1 h (*see* **Note 6**).
5. Add 20 µL of DEPC-treated ddH_2O and mix well.
6. Add a mixture of 2 µL 0.5 M EDTA and 22 µL 7.5 M ammonium acetate. Mix well.
7. Purify the synthesized ssRNA with 1 vol of acidic phenol–chloroform–isoamylalcohol; subsequently, extract 1 vol of chloroform–isoamylalcohol.
8. Precipitate with 2.5 vol of 100% ethanol for at least 1 h at –80 °C.
9. Centrifuge for 30 min at 4 °C and 20,000 x g.
10. Wash the RNA pellet with 70% ethanol and spin down.
11. Air-dry the pellet.
12. Dissolve the ssRNA in 20 µL of DEPC-treated PBS (*see* **Notes 6** and **7**).
13. Mix equal ng amounts of antisense and sense ssRNAs (*see* **Note 8**).
14. Heat the mixture for 5 min at 95 °C, and allow for it to cool down slowly to room temperature by switching off the heating block (*see* **Note 6**).
15. Confirm the proper annealing by gel electrophoresis (*see* **Note 6**).
16. Store the dsRNA at –80 °C until further use (*see* **Note 17**).

3.2. Windowing the Eggs

For access to the embryo, the eggs are windowed on the third day of incubation (**Figs. 1a** and **2**).

1. Incubate the fertilized eggs in a preincubator at 39 °C (*see* **Notes 9** and **10**).
2. After three days of incubation, place the egg on the side for at least 30 min before opening, to allow the embryo to reposition on top of the yolk.
3. Mark the position of the embryo on the egg shell with a pencil using an Egg-Lume Candler held against the blunt end of the egg.
4. Wipe the eggshell with 70% ethanol to avoid contamination.
5. Make small holes at the blunt end of the egg and at the corners, outlining the planned window using a scalpel (**Fig. 2a**).
6. Carefully remove 3 mL of albumin through the hole at the blunt end of the egg using a syringe (**Fig. 2a**; *see* **Note 11**).
7. Seal the hole at the blunt end and any possible cracks in the shell by applying melted paraffin.
8. Put a piece of Scotch tape onto the shell to prevent small pieces of the eggshell from falling into the egg (**Fig. 2a**).
9. Cut the outlined window into the eggshell (*see* **Note 12**).
10. Seal the egg by applying melted paraffin to the edges of the window using a brush and a coverslip (**Fig. 2b**; *see* **Note 13**).

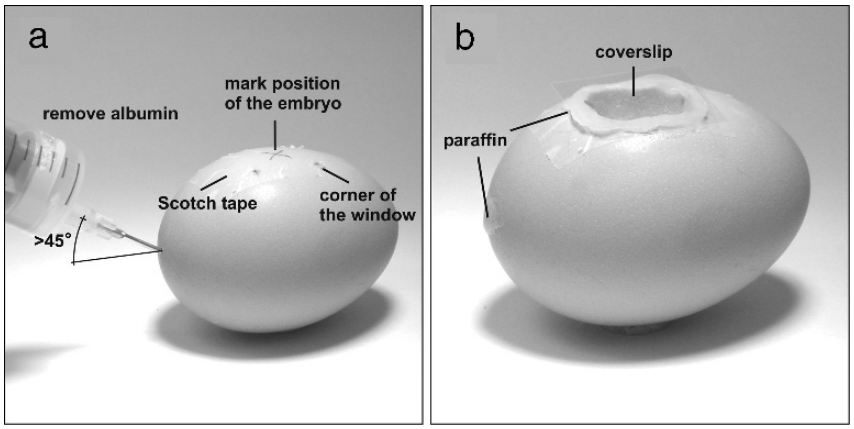


Fig. 2. Windowing the egg. (a) Before egg windowing, the position of the embryo is marked with a pencil on the shell. Subsequently, small holes are drilled at the blunt end of the egg and at the corners outlining the window. Albumin (3 mL) is removed through the hole at the blunt end (*see* **Note 11**). The syringe is kept at a 45º angle to avoid damage to the embryo and the yolk. A piece of Scotch tape prevents pieces of the shell from falling inside the egg while cutting the window with small scissors. (b) For further incubation, the window is sealed with melted paraffin and a coverslip.

11. Put the egg back in the incubator. Make sure that the position of the egg is the same as before windowing to keep the embryo accessible through the window (*see* **Note 17**).

3.3. In ovo *Injection and Electroporation*

1. Autoclave the tools and wipe the working space with 70% ethanol.
2. Reopen the sealed egg by pressing the soldering iron briefly onto the coverslip and removing it carefully.
3. Stage the embryo according to Hamburger and Hamilton ***(21)***. Embryos should be between stages 17 and 19.
4. Remove the extraembryonic membranes covering the embryo with forceps and scissors in order to have direct access to the embryo (**Fig. 3a**).
5. Break off the needle tip to obtain a tip diameter of approximately 5 µm.
6. Sterile PBS containing the dsRNA derived from AXONIN-1 (200–400 ng/µL) and an EGFP reporter plasmid (20 ng/µL) with 0.04 % (vol/vol) Trypan Blue are injected into the central canal of the spinal cord at the level of the hind limbs using a glass capillary connected to a piece of tubing (**Figs. 1c** and **3b**). The injection is controlled by mouth, and the maximal injection volume is achieved when the blue dye reaches the brain vesicle (arrowhead in **Fig. 3b**).
7. Add a few drops of PBS before electroporation to lower the electric resistance and to prevent overheating of the embryo.
8. The electrodes are placed in a parallel manner along the anterior-posterior axis of the spinal cord (**Fig. 3c**).
9. Electroporate the embryo by applying five pulses of 26 V and 50-ms duration each (*see* **Note 14**).
10. After the electroporation, add a few drops of PBS to cool the embryo.
11. Rinse the electrodes with plenty of distilled water to remove denaturated proteins from the surface (*see* **Note 15**).
12. Reseal the egg with a glass coverslip and a soldering iron (*see* **Note 13**).
13. For further incubation, the egg is returned to 39 °C until stage 25 is reached, i.e., approximately two additional days of incubation ***(21)*** (*see* **Note 17**).

3.4. Analysis of the Phenotype and Electroporation Efficiency

For beginners we recommend assessing the efficiency and reproducibility of the *in ovo* electroporation by analyzing EGFP expression directly *in ovo* under the stereomicroscope (**Fig. 3d**). Thus, embryos for further analyses can be preselected (*see* **Note 16**).

The efficiency as well as the specificity of gene silencing by *in ovo* RNAi can be demonstrated by a variety of approaches. Immunohistochemistry on cryostat sections ***(4,13,22)*** and Western blot analysis ***(23,24)*** are common ways to show downregulation of the targeted protein. If antibodies against the targeted

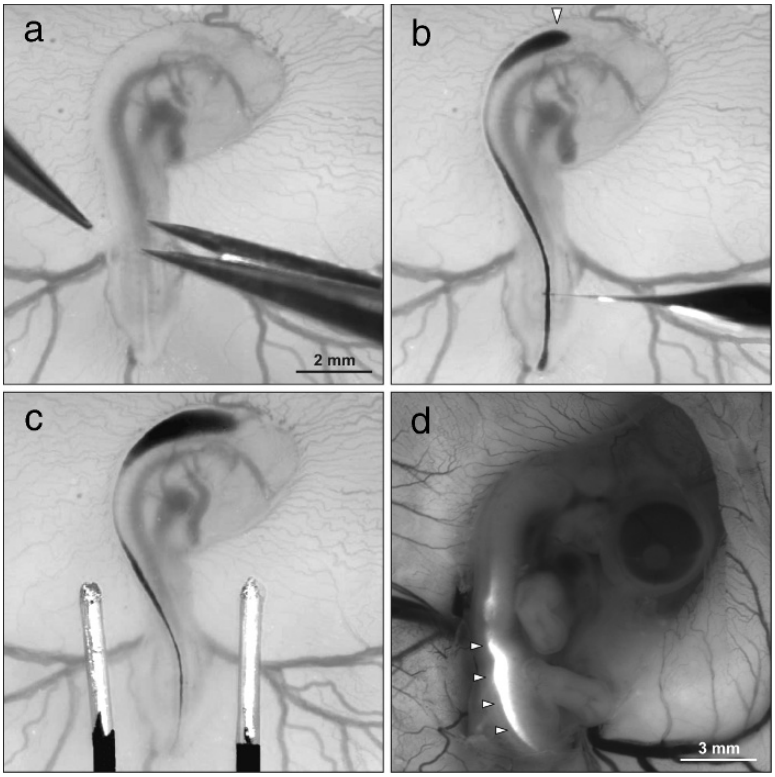


Fig. 3. Injection and electroporation of the embryo. (a) The extraembryonic membranes covering the embryo are carefully removed before injection using forceps and spring scissors. The injection mixture containing dsRNA derived from the gene of interest, an EGFP plasmid for transfection control, and Trypan Blue is injected into the central canal with a glass capillary. The maximal injection volume is achieved when the Trypan Blue has reached the brain vesicle [arrowhead in (b)]. (c) After retraction of the injection needle, the electrodes are placed in a parallel manner along the embryonic axis. For stage 18 embryos, five pulses of 26 V with 50-ms duration are applied for efficient transfection (*see* **Note 14**). (d) The successful transfection can be verified by the expression of EGFP (indicated by arrowheads) under a stereomicroscope equipped with fluorescence optics two days after electroporation.

protein are not available, a decrease in the mRNA can be assessed by *in situ* hybridization using either whole-mount embryos ***(16,25)*** or cryostat sections ***(13)***. Alternatively, semi-quantitative RT-PCR can be used to detect a decrease in the mRNA ***(26)***.

Loss-of-function phenotypes can be analyzed in a variety of ways. To study cell differentiation or cell migration, immunohistochemistry for known

markers may be a good start ***(15)***. Changes in morphology and cell positions, expression patterns, etc. can easily be detected. To visualize axonal trajectories, staining and/or dye tracing in slices or whole-mount preparations are used to account for their three-dimensionality. For an initial assessment and to localize a specific phenotype within the peripheral nervous system, we recommend a neurofilament staining of whole-mount preparations ***(24)***. Alternatively or subsequently, mechanisms involved in wiring the nervous system can be analyzed in vibratome slices by dye tracing or immunohistochemistry ***(4,14)***. For example, we studied molecular mechanisms underlying the pathfinding behavior of commissural axons in the spinal cord using dye tracing in open-book preparations ***(4,13,27)***.

4. Notes

1. Different recipes exist for PBS, and the addition of KCl turned out to be crucial for optimal survival rates.
2. The incubation time and developmental progress of the embryo are dependent on the temperature and humidity. Any incubator set at a temperature of 38–39 °C can be used, as long as high humidity (at least 45%) and good air circulation can be achieved. The use of two incubators, one for preincubation and one for treated embryos, is recommended to minimize contamination and to reduce detrimental effects on the treated embryos due to frequent opening and closing of the incubator.
3. Alternatively, any other electroporator that generates square wave pulses can be used (for a comparison of the different electroporators that are commercially available, *see* **Ref. *8***). For different target tissues, different electrodes have to be chosen to get best results: For *in ovo* electroporations of the developing spinal cord, we use wire electrodes ***(4,13)***. Commercially available electrodes can be found at http://www.btxonline.com/products/electrodes/inovo. Alternatively, for a widespread transfection, platelet electrodes can be used ***(28)***. For a small transfection area, a needle electrode can be placed directly into the tissue ***(17,29,30)***.
4. In addition to long dsRNA, short interfering RNAs (siRNA) and short hairpin RNAs (shRNA) have been applied successfully for RNAi in chicken embryos ***(14–17,22,31)***. In contrast to siRNAs selected by various available algorithms, long dsRNA always effectively silenced target genes in our hands. Long dsRNA is processed by Dicer to give rise to a large number of siRNAs and therefore will always produce many effective ones, making lengthy (and expensive) selection processes unnecessary. Furthermore, long dsRNA can easily be produced by *in vitro* transcription from a cDNA fragment or EST without further cloning steps or expensive synthesis of siRNAs. Chicken ESTs can be obtained from Geneservices Ltd. at http://www.geneservice.co.uk. To exclude any off-target effects

(silencing of nontarget genes), we recommend using two different nonoverlapping dsRNA fragments, as it is highly unlikely that they would both have the same off-target effects. In contrast to mammalian cell lines and nonembryonic tissue, long dsRNA can be applied to embryonic tissue without induction of unspecific effects ***(4,18,19)***. No general inhibition of protein synthesis or induction of apoptosis has been reported in mouse oocytes, embryo-derived cell lines, and chicken embryos ***(4,15,32,33)***.

5. For *in vitro* transcription, RNase-free tips, tubes, and DEPC-treated ddH_2O have to be used. Before starting, clean the workspace with RNaseZAP.
6. Collect 1-µL samples after each step and keep them to control the quality of ssRNA and dsRNA. For this purpose, load the ssRNAs collected after each step and the dsRNA on a 1% agarose gel. Lanes 1 and 2 (one sense and one antisense) contain the linearized plasmids and the ssRNA synthesized by *in vitro* transcription. Lanes 3/4 and 5/6 are sense and antisense ssRNA after DNaseI treatment and purification, respectively. In lane 7 the resulting dsRNA after annealing is loaded. When lanes 5 and 6 are compared to lane 7, the band shift due to the difference in migration between ssRNA and dsRNA should be detected. Furthermore, both ssRNA and dsRNA should give distinct bands. If a smear indicating degradation of the RNA is obtained, the dsRNA should not be used for *in ovo* RNAi.
7. Make sure that the pH and salt concentration of the buffer used to dissolve the ssRNA are in the physiological range and do not have any effect on the development and survival rate of the embryo. Do not use any buffers containing Tris or glycerol.
8. The concentration of the dsRNA for *in vivo* injections should be in the range of 200–400 ng/µL.
9. Eggs should be stored at 15 °C for a maximum of one week before incubation. When the eggs are stored for longer periods, normal development of the embryo is unlikely.
10. To reach 45% humidity, it is usually sufficient to place a tray of distilled water containing 0.1 g/L of copper sulfate at the bottom of the incubator. Copper sulfate decreases the risk of contamination.
11. The holes at the corners are required to allow entry of air and detachment of the embryo from the shell during removal of albumin. Insert the syringe at a steep angle (about 45°; **Fig. 2a**) to avoid damage to the embryo and the yolk that is not compatible with survival.
12. Keep the scissors as horizontally as possible to prevent any damage to the embryo.
13. Make sure that the window is properly closed to prevent dehydration during further incubation. Dehydration will cause the embryo's death. If the paraffin is cooled down too quickly, heat the coverslip briefly with the soldering iron while pressing it down so that the coverslip seals properly along all the edges. Alternatively, the window can be sealed with Scotch tape. Although sealing with coverslips instead of tape is more time-consuming, it facilitates reopening the

window and the development of the embryo can be directly observed through the coverslip. The window can easily be reopened by brief heating of the coverslip using a soldering iron.

14. The electroporation settings should be chosen according to the embryonic stage (also *see* **Ref. *12***): For stage 18 embryos, 5 pulses of 26 V and 50 ms are applied. The voltage should be adjusted to the embryonic stage and the tissue that is electroporated:

Day of Incubation	Embryonic Stage	Target Tissue	Settings
2	12–14*	Spinal cord and neural crest derivates (for example, dorsal root ganglia)	18 V
3	18–20**	Spinal cord and floor plate	26 V

* Red ink is applied to visualize the embryo. Blue ink should not be used, because it interferes with detection of the Trypan Blue that is used to control injection volume and injection site.

** Electroporation at stage 20 or later with the settings mentioned here will prevent transfection of lateral motor neurons.

During electroporation, contact between the electrodes and the major blood vessels as well as with the embryo has to be avoided to prevent severe damage to, or the death of, the embryo. Keep the electrodes away from the heart.

15. Remaining proteins on the electrodes interfere with efficient electroporation.
16. The transfection efficiency depends on the time point of the injection, the concentration of the injected nucleic acids, and the electroporation settings. *In ovo* electroporation with the given settings at embryonic stage 18 resulted in a transfection efficiency of approximately 60% of cells within the electroporated area ***(4)***.
17. Troubleshooting list:

Protocol Step	Potential Problem	Troubleshooting: See Note
Synthesis of long dsRNA	Degradation or bad quality of dsRNA	5, 6
Windowing the eggs	Low survival rate	9, 11, 12, 13
	Contamination	2, 10
	Delay in development	2, 9, 13
In ovo injection and electroporation	Low survival rate	1, 3, 7, 8, 13, 14
	Contamination	2, 10
	Inefficient transfection	14, 15, 16

References

1. Calegari, F., Marzesco, A. M., Kittler, R., Buchholz, F., and Huttner, W. B. (2004). Tissue-specific RNA interference in post-implantation mouse embryos using directional electroporation and whole embryo culture. *Differentiation* **72,** 92–102.
2. Takahashi, M., Sato, K., Nomura, T., and Osumi, N. (2002). Manipulating gene expressions by electroporation in the developing brain of mammalian embryos. *Differentiation* **70,** 155–162.
3. Bai, J., Ramos, R. L., Ackman, J. B., Thomas, A. M., Lee, R. V., and LoTurco, J. J. (2003). RNAi reveals doublecortin is required for radial migration in rat neocortex. *Nat. Neurosci*. **6,** 1277–1283.
4. Pekarik, V., Bourikas, D., Miglino, N., Joset, P., Preiswerk, S., and Stoeckli, E. T. (2003). Screening for gene function in chicken embryo using RNAi and electroporation. *Nat. Biotechnol*. **21,** 93–96.
5. Baeriswyl, T., and Stoeckli, E. T. (2006). *In ovo* RNAi opens new possibilities for temporal and spatial control of gene silencing during development of the vertebrate nervous system. *J. RNAi Gene Silencing* **2,** 126–135.
6. Bourikas, D., and Stoeckli, E. T. (2003). New tools for gene manipulation in chicken embryos. *Oligonucleotides* **13,** 411–419.
7. Prawitt, D., Brixel, L., Spangenberg, C., Eshkind, L., Heck, R., Oesch, F., Zabel, B., and Bockamp, E. (2004). RNAi knock-down mice: An emerging technology for post-genomic functional genetics. *Cytogenet. Genome Res*. **105,** 412–421.
8. Krull, C. E. (2004). A primer on using *in ovo* electroporation to analyze gene function. *Dev. Dyn*. **229,** 433–439.
9. Momose, T., Tonegawa, A., Takeuchi, J., Ogawa, H., Umesono, K., and Yasuda, K. (1999). Efficient targeting of gene expression in chick embryos by microelectroporation. *Dev. Growth Differ*. **41,** 335–344.
10. Muramatsu, T., Mizutani, Y., Ohmori, Y., and Okumura, J. (1997). Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos *in ovo*. *Biochem. Biophys. Res. Commun*. **230,** 376–380.
11. Nakamura, H., and Funahashi, J. (2001). Introduction of DNA into chick embryos by *in ovo* electroporation. *Methods* **24,** 43–48.
12. Itasaki, N., Bel-Vialar, S., and Krumlauf, R. (1999). "Shocking" developments in chick embryology: Electroporation and *in ovo* gene expression. *Nat. Cell Biol*. **1,** E203–E207.
13. Bourikas, D., Pekarik, V., Baeriswyl, T., et al. (2005). Sonic hedgehog guides commissural axons along the longitudinal axis of the spinal cord. *Nat. Neurosci*. **8,** 297–304.
14. Bron, R., Eickholt, B. J., Vermeren, M., Fragale, N., and Cohen, J. (2004). Functional knockdown of neuropilin-1 in the developing chick nervous system by siRNA hairpins phenocopies genetic ablation in the mouse. *Dev. Dyn*. **230,** 299–308.
15. Chesnutt, C., and Niswander, L. (2004). Plasmid-based short-hairpin RNA interference in the chicken embryo. *Genesis* **39,** 73–78.

16. Katahira, T., and Nakamura, H. (2003). Gene silencing in chick embryos with a vector-based small interfering RNA system. *Dev. Growth Differ*. **45,** 361–367.
17. Toyofuku, T., Zhang, H., Kumanogoh, A., et al. (2004). Guidance of myocardial patterning in cardiac development by Sema6D reverse signalling. *Nat. Cell Biol*. **6,** 1204–1211.
18. Svoboda, P. (2004). Long dsRNA and silent genes strike back: RNAi in mouse oocytes and early embryos. *Cytogenet. Genome Res*. **105,** 422–434.
19. Gan, L., Anton, K. E., Masterson, B. A., Vincent, V. A., Ye, S., and Gonzalez-Zulueta, M. (2002). Specific interference with gene expression and gene function mediated by long dsRNA in neural cells. *J. Neurosci. Meth*. **121,** 151–157.
20. Hillier, L. W., Miller, W., Birney, E., et al. (2004). Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature* **432,** 695–716.
21. Hamburger, V., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol*. **88,** 49–92. Reprinted as Sanes, J. R. (1992). On the republication of the Hamburger–Hamilton stage series. *Dev. Dyn.* **195,** 229–272.
22. Rao, M., Baraban, J. H., Rajaii, F., and Sockanathan, S. (2004). *In vivo* comparative study of RNAi methodologies by *in ovo* electroporation in the chick embryo. *Dev. Dyn*. **231,** 592–600.
23. Bourikas, D., Baeriswyl, T., Sadhu, R., and Stoeckli, E. T. (2005). *In ovo* RNAi opens new possibilities for functional genomics in vertebrates. In *RNA Interference Technology—From Basic Research to Drug Development* (K. Appasani, ed.), Cambridge University Press, Cambridge, UK, pp. 220–232.
24. Stepanek, L., Stoker, A. W., Stoeckli, E., and Bixby, J. L. (2005). Receptor tyrosine phosphatases guide vertebrate motor axons during development. *J. Neurosci*. **25,** 3813–3823.
25. Dai, F., Yusuf, F., Farjah, G. H., and Brand-Saberi, B. (2005). RNAi-induced targeted silencing of developmental control genes during chicken embryogenesis. *Dev. Biol*. **285,** 80–90.
26. Sato, F., Nakagawa, T., Ito, M., Kitagawa, Y., and Hattori, M. A. (2004). Application of RNA interference to chicken embryos using small interfering RNA. *J. Exp. Zoolog. A Comp. Exp. Biol*. **301,** 820–827.
27. Perrin, F. E., and Stoeckli, E. T. (2000). Use of lipophilic dyes in studies of axonal pathfinding *in vivo*. *Microsc. Res. Tech*. **48,** 25–31.
28. Matsuda, T., and Cepko, C. L. (2004). Electroporation and RNA interference in the rodent retina *in vivo* and *in vitro*. *Proc. Natl. Acad. Sci. USA* **101,** 16–22.
29. Oberg, K. C., Pira, C. U., Revelli, J. P., Ratz, B., Aguilar-Cordova, E., and Eichele, G. (2002). Efficient ectopic gene expression targeting chick mesoderm. *Dev. Dyn*. **224,** 291–302.
30. Luo, J., and Redies, C. (2004). Overexpression of genes in Purkinje cells in the embryonic chicken cerebellum by *in vivo* electroporation. *J. Neurosci. Meth*. **139,** 241–245.

31. Nakamura, H., Katahira, T., Sato, T., Watanabe, Y., and Funahashi, J. (2004). Gain- and loss-of-function in chick embryos by electroporation. *Mech. Dev*. **121,** 1137–1143.
32. Billy, E., Brondani, V., Zhang, H., Muller, U., and Filipowicz, W. (2001). Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc. Natl. Acad. Sci. USA* **98,** 14428–14433.
33. Stein, P., Zeng, F., Pan, H., and Schultz, R. M. (2005). Absence of non-specific effects of RNA interference triggered by long double-stranded RNA in mouse oocytes. *Dev. Biol*. **286,** 464–471.

7. Discussion

The aggregation and formation of BCC clusters are crucial steps during the establishment of the nervous system. They maintain the integrity of the CNS and the PNS and control the maturation of the DREZ. Furthermore, BCCs possess stem cell-like characteristics that are important for the contribution to PNS structures, such as nociceptive neurons and peripheral glia. In my thesis I propose an interaction between Semaphorin6A and PlexinA1 that mediates the aggregation of ventral BCCs at the VMEP. Furthermore, in the absence of either Semaphorin6A or PlexinA1 the dorsal BCC clusters were malformed or even absent. However, since the dorsal BCCs aggregate before the ingrowth of primary sensory axons into the spinal cord, the aggregation mechanism of dorsal clusters cannot be compared to the ventral ones. Further studies will have to elucidate whether other extrinsic factors present in the ECM or in the neuroepithelium are responsible for the aggregation of dorsal BCCs. In the absence of ventral BCCs, motor neurons are leaving the spinal cord along the ventral roots. It is unknown, however, whether they are capable of surviving outside of the motor column. This may explain why translocated motor neurons were mainly reported in the proximal ventral root and never at a greater distance from their origin (Vermeren et al., 2003). Similarly, in the absence of dorsal BCCs up to 50% of nociceptive neurons were missing in the DRGs and the peripheral glia was markedly reduced (Maro et al., 2004).
Even though many aspects of BCCs are unknown, understanding their formation, contribution, and persistence in the nervous system might provide crucial information for future therapeutic treatments.

Sox8 and Semaphorin6A appear to be in the same pathway

The homeobox gene Sox8 is involved in the induction of NC cells and was shown to play a role during the late phase of oligodendrocyte differentiation (Stolt et al., 2004). Interestingly, Semaphorin6A shows a very similar expression pattern during early embryogenesis: both are initially expressed in the dorsal neural tube, later in BCCs, and in the ventral ventricular zone, the region where oligodendrocyte precursors are formed (Bell et al., 2000; Cheung and Briscoe, 2003; Mauti et al., 2007). Sox8 was shown to bind to the same response elements as Sox10 does, and thus, Sox10 mutant mice carrying Sox8 in the Sox10 locus can partially compensate for the loss of Sox10 (Stolt et al., 2004; Kellerer et al., 2006). In a yeast-two hybrid screen Sox8 and Sox10 were shown to interact with many transcription factors such as Krox20, Olig2, Pax3, and others (Wissmüller et al., 2006). However, no direct link between Sox8 and Semaphorin6A was reported so far, but the precise overlap among both genes disserves further attention.

A Semaphorin-Plexin interaction is responsible for the aggregation of ventral BCCs

Based on our results we propose the following mechanism for the aggregation of BCCs at the VMEP of the lumbar spinal cord: Semaphorin6A, which is expressed by NC cells that give rise to BCCs, binds to PlexinA1 present on motor axons. This interaction arrests the migration of NC cells via

reverse Semaphorin6A signaling and initiates the formation of the ventral BCC clusters. This hypothesis is supported by several findings: (1) A recent ultrastructural study revealed that the ventral BCCs appear at the VMEP after the outgrowth of the first motor axons (Fraher et al., 2007). This study shows clearly that no BCCs are present at the VMEP at any axial level during the initial motor axon outgrowth, and thus, that they do not define the VMEP (see Vermeren et al., 2003). (2) The intracellular domain of Semaphorin6A associates with Ena/VASP proteins that were shown to modulate actin cytoskeleton dynamics of migrating cells (Klostermann et al., 2000; Drees and Gertler, 2008). (3) Class-6 Semaphorins were already shown to control the migration of crest cells: the migration of endocardiac crest cells is controlled by Semaphorin6D (Toyofuku et al., 2004). (4) Chicken Semaphorin6A and PlexinA1 where shown to interact (Shanthini Sockanathan, personal communication). (5) Blocking PlexinA1 on motor axons by ectopic expression of the transmembrane and soluble form of Semaphorin6A results in malformed BCC clusters and thus, to motor neuron translocations (Mauti et al., 2007). (6) Overexpression of Shh in the spinal cord enlarges the motor column resulting in an extended motor neuron pool with multiple ventral roots (data not shown). In such embryos, all roots were populated by BCCs expressing Semaphorin6A and Krox20 (data not shown). This finding supports the hypothesis that motor axons initiate the aggregation of BCCs.
It is noteworthy, that HB9-mutant mice exhibit a similar motor neuron translocation phenotype as observed in the absence of BCCs or Semaphorin6A (Arber et al., 1999). HB9, a homeobox gene required for the specification of motor neurons, is likely to induce and regulate the expression of PlexinA1 which is needed for the aggregation of BCCs. The authors did not investigate whether the BCC clusters were affected as one would predict according to our model, where the failure to express PlexinA1 on motor neurons would result in malformed BCC clusters, and thus, to motor neuron emigration.

Why do ventral boundary cap cells have to stabilize the motor column?

Motor neurons derive from a highly proliferative zone (pMN) in the ventral ventricular zone of the neural tube. The postmitotic motor neuron precursors migrate and mature on the way to their final position in the ventral spinal cord. During the migration process, they start to send out axons into the periphery (Bron et al., 2007). One important question arises from this observation: why are the first motor neurons not translocating into the periphery prior to the aggregation of ventral BCCs? Three possible assumptions could explain this finding. First, the basal lamina could form an initial (weak) physical barrier that prevents the first motor neuron cell bodies, but not the motor axons, to exit the spinal cord. However, the progressive maturation of motor neurons and thus, the increased perforation of the initial barrier by axons makes it more permissive for somal translocations. This is the time point when the ventral BCCs are formed and take over the primary barrier function of the basal lamina. Second, the motor axon elongation is much faster compared to the motor neuron migration. Thus, when the motor neuron somas reach the outer border of the spinal cord, the BCCs are already aggregated and stabilize the motor column. Third, cell adhesion molecules expressed on early born motor neurons could initially prevent their translocation. Indeed, Price and coworkers found that MN-

Cadherin is present in postmitotic motor neurons and is involved in sorting of motor neurons within the motor column (Price et al., 2002). However, the authors did not report ectopic motor neurons after inactivation of MN-Cadherin. It is therefore likely that the basal lamina can initially stabilize the early born motor neurons but this weak barrier needs to be replaced by a stronger one during the progressive development of motor neurons.

Stabilization of the motor column

Recently, Bron and coworkers discovered a mechanism that possibly arrests the migration of motor neurons in the prospective motor column (Bron et al., 2007). The interaction of BCC-derived Semaphorin6A with PlexinA2 on motor axons was shown to prevent motor neuron translocations, as in the absence of either Semaphorin6A (see above) or PlexinA2, motor neuron somas were leaving the spinal cord. The authors further linked MICAL3 (molecule interacting with CasL, type 3), which was shown to bind intracellularly to PlexinAs (Terman et al., 2002), to the Semaphorin-Plexin interaction that possibly arrests somal migration of motor neurons. However, the suggested hypothesis of the Semaphorin-Plexin-MICAL3 stabilization of the motor column contradicts our finding in a very interesting point: we showed that ectopic expression of Semaphorin6A (soluble and transmembrane form) in motor neurons resulted in the translocation of motor neurons by inhibiting the aggregation of BCCs. According to Bron and coworkers, the exposure of motor neurons to Semaphorin6A should arrest the migration which is in disagreement with our findings (compare Bron et al., 2007; Mauti et al., 2007). Furthermore, the authors claim that the interaction among Semaphorin6A and PlexinA2 occurs in the lumbosacral region of the chicken embryo, and as a consequence, the Semaphorin6A knockout mouse preferentially shows ectopic motor neurons in the hind limb region. An important point which the authors might have missed is that the expression patterns of Plexins are very dynamic along the anterior-posterior axis and during embryogenesis (Mauti et al., 2006). We were unable to detect significant expression levels of PlexinA2 in the lumbosacral motor column, but instead, very strong expression of PlexinA1. Conversely, the thoracic motor column expressed PlexinA2 whereas PlexinA1 was present at moderate levels. PlexinA4 was not present in motor neurons during the initial motor axon outgrowth. Thus, the discrepancies between both studies appear to be due to different anterior-posterior levels of the analyzed embryos. This further suggests that other PlexinAs and/or Semaphorins are involved in the establishment and the function of BCCs along the anterior-posterior axis. Indeed, in contrast to mice (Yoshida et al., 2006), all PlexinAs were shown to bind to Semaphorin6A (Shanthini Sockanathan, personal communication) and it will be worthwhile to analyze whether all PlexinAs could induce the aggregation of BCCs.

Do ventral boundary cap cells organize the motor column?

Motor neurons derive from a common progenitor cell in the ventral ventricular zone and differentiate into two distinct pools; the MMC and the LMC (see Figure 3). For example, early born motor neurons

form the LMC_M, whereas late born motor neurons acquire LMC_L identity (Jessell, 2000). The LMC_M motor neurons were shown to secrete retinoic acid which induces the LMC_L identity of late born motor neurons (Sockanathan and Jessell, 1998). The position of the motor neuron soma within the motor column was shown to be linked to target muscle innervation (Livet et al., 2002). Thus, the emigration of motor neurons in the absence of BCCs could even potentiate motor neuron defects: initial loss of LMC_M motor neurons leads to a failure to induce and specify the late born motor neurons of the LMC_L pool and hence to target innervation failures due to a lack of neurons in both LMC compartments. Considering this, it is likely that ventral BCCs are necessary to organize the motor column. Alternatively, the motor column could compensate for the loss of certain motor neurons, and thus prevent severe innervation defects. Further analyses on the organization of the motor column and target muscle innervations in absence of ventral BCCs will have to address these questions.

Evidence for multiple mechanisms in the stabilization of the motor column

Motor neurons were shown to leave the spinal cord in the absence of ventral BCCs. It is unknown whether the emigration is subpool specific or whether all motor neurons could translocate. Interestingly, absence of Semaphorin6A (in mice and chicken), genetic ablation of BCCs (Krox20-DT mice) or lack of BCCs in *Splotch* mice resulted in a less severe translocation of motor neurons compared to the surgical ablation of the NC (compare Vermeren et al., 2003; Bron et al., 2007; Mauti et al., 2007). How can these different intensities of the motor neuron translocation phenotype be explained? The ablation of the NC results in a complete absence of PNS structures, including BCCs and peripheral glia. Similarly, *Splotch* mice have a strongly reduced number of NC cells in the trunk, but importantly, the NC cells are not completely absent, their formation is markedly delayed (Moase and Trasler, 1990). This implies that the formation of some PNS cell types, i.e. peripheral glia, takes place to a certain extent. Indeed, glial cells were occasionally observed along ventral roots of *Splotch* mice (Grim et al., 1992). In contrast to previous reports, these cells must be NC-derived and cannot emigrate directly from the ventral neural tube (compare Le Douarin et al., 1991; Vermeren et al., 2003). Krox20-DT mice lack BCCs and partially ventral root glial cells whereas the absence of Semaphorin6A results in the failure to form BCCs and as a consequence, the motor column is destabilized (Vermeren et al., 2003; Maro et al., 2004; Mauti et al., 2007). In both cases, a similar intensity of the motor neuron translocation phenotype was observed. Analyzing Semaphorin6A-deficient chicken embryos at older stages revealed a similarly restricted loss of motor neurons as found at earlier stages (data not shown). Interestingly, MICAL3 is present in the entire motor column and its downregulation results in a comparably strong motor neuron translocation phenotype as seen after the ablation of the NC (compare Vermeren et al., 2003; Bron et al., 2007). Despite these findings the stabilization of the motor column could be not solely dependent on BCCs, but also on peripheral glia. Since peripheral glial cells aggregate after BCCs, I propose the following refined working hypothesis (see Figure 7): Initially, the first motor neurons are confined to the spinal cord by a (weak) basal lamina-derived barrier. Subsequently, the BCCs are formed that stabilize the early motor

neurons, possibly LMC_M neurons. The later born motor neurons get induced by LMC_M neurons to acquire LMC_L identity and are stabilized by a peripheral glial cell-derived mechanism.

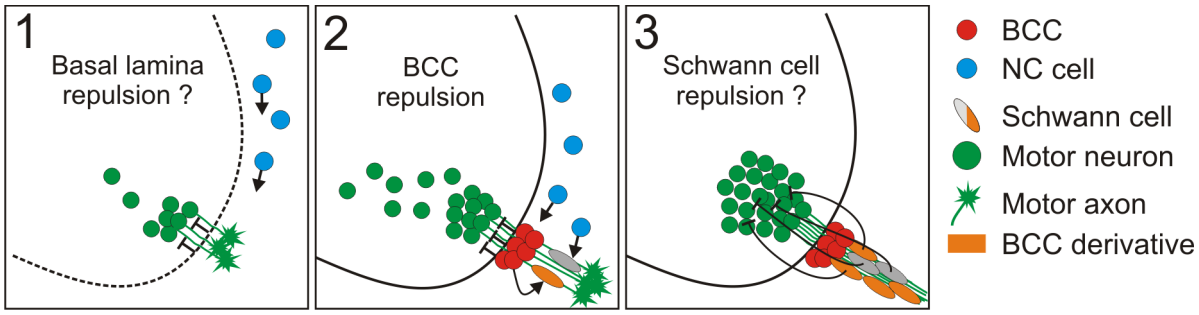


Figure 7: Multiple mechanisms could contribute to the motor column stabilization.
(1) The basal lamina retains early born motor neurons in the spinal cord. (2) Subsequently, more postmitotic motor neurons arrive at the motor column and extend axons into the periphery. This is the time point when the ventral BCCs aggregate and stabilize a larger number of motor neurons. The Schwann cells of the ventral roots (oval) derive partially from BCCs (orange) but also from NC cells (grey). (3) Finally, the latest born motor neurons are stabilized by peripheral glia cells-derived factors.

Further evidence for a peripheral glia cell-derived stabilization mechanism is provided by Vermeren and coworkers (Vermeren et al., 2003): quail NC cell grafts in NC-ablated avian embryos appear to aggregate preferentially along the roots and not specifically at the VMEP, suggesting that they rather form peripheral glial cells than BCCs. Unfortunately, the authors did not analyze the identity of the aggregated NC cells. In these embryos the integrity of the motor column is largely rescued, since only a few ectopic motor neurons could be detected. It is possible that the emigrated motor neurons are the early born LMC_M neurons that failed to be arrested by BCCs. However, the identity of the translocated motor neurons was never investigated in more details, and thus, disserves further attention in future studies.

Why would there be a need for such multiple mechanisms? The simplest explanation is a redundancy to ensure that the stabilization of the motor column is not dependent on the function of only one gene (Semaphorin6A). Interestingly, the innervation pattern of motor axons in *Splotch* mice was reported to be mainly unaffected, even though the axons grew slower (Grim et al., 1992). This could possibly indicate a compensation mechanism for the loss of neurons in the motor column (see above).

Clustering of ventral BCCs

Shortly after BCCs aggregate at the transition zones they express various cell adhesion molecules such as N-Cadherin, Cadherin-7, and P_0 (Nakagawa and Takeichi, 1995; Golding and Cohen, 1997; Wanner et al., 2006). These molecules have often multiple functions, as seen for N-Cadherin which is a cell adhesion molecule but can also promote neurite outgrowth (Bixby and Zhang, 1990). P_0 has also been implicated in cell adhesion and neurite outgrowth but is also a component of peripheral myelin (Filbin et al., 1990; Schneider-Schaulies et al., 1990). Among these molecules, the Cadherins are the most likely candidates for mediating the adherence of BCCs to axons and themselves.

Cadherin-7 is expressed in various regions of the spinal cord such as motor neurons, intermediate ventricular zone and migrating NC cells (Nakagawa and Takeichi, 1995; Luo et al., 2006). Interestingly, the Semaphorin6A-positive NC cells (or BCC precursors) do not colocalize with Cadherin-7-positive cells, suggesting that Cadherin-7 is upregulated once they aggregate (compare Nakagawa and Takeichi, 1995; Mauti et al., 2007). In the spinal cord, Cadherin-7 was reported to be regulated by both, Shh and the homeobox gene Pax7 (Luo et al., 2006): low levels of Shh activate Cadherin-7, while high levels repress it. An indicator for low Shh concentration is the upregulation of Gli3, a mediator of the Patched/Smoothened pathway, which is activated in the dorsal neural tube. Furthermore, Pax7 as a negative regulator of Cadherin-7 restricts its expression to the intermediate zone. BCCs were shown to express Gli3 and Pax7 but in contrast to the dorsal neural tube, they express Cadherin-7 (Luo et al., 2006). The presence of Gli3 in BCCs probably indicates a low Shh concentration derived from the notochord which spreads throughout the ECM. It is likely that the expression level of Cadherin-7 in BCCs is dynamically regulated by a mechanism involving Shh and Pax7, ensuring constant expression levels of Cadherin-7.

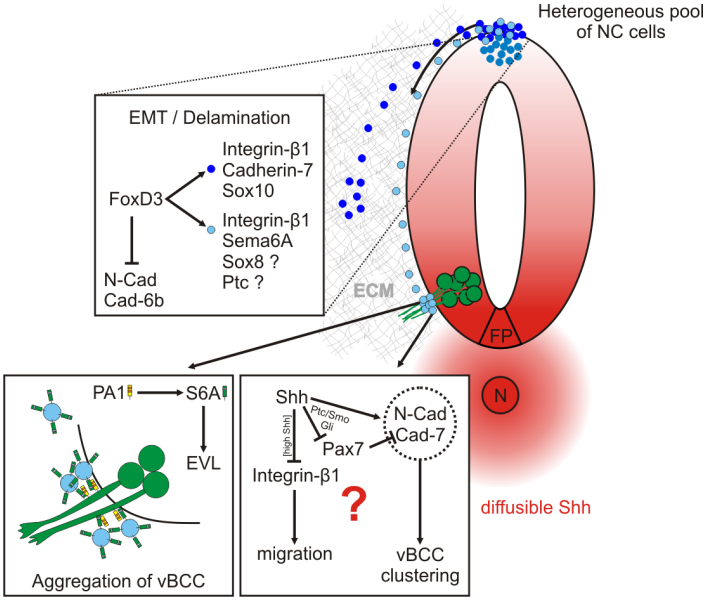


Figure 8: Proposed model for the clustering of ventral BCCs.
NC cells derive from a heterogeneous pool of cells in the dorsal neural tube. The precursors of BCCs (light blue) are supposed to be distinct in the expression of certain genes compared to the remaining migrating NC cells (dark blue). However, both pools express Integrin-β1 which is necessary for the migration through the ECM or along the basal lamina of the spinal cord. Sonic Hedgehog (Shh, red) is secreted from the notochord (N) and the floor plate (FP) and is supposed to be distributed in the ECM with the help of HSPGs. BCCs were shown to express Gli3 and there is evidence for the presence of Patched (data not shown). Shh is supposed to control the expression of Cadherin-7 and N-Cadherin via the Patched/Smoothened/Gli pathway, and at least for Cadherin-7, Pax7 is a known repressor. High Shh concentrations were shown to block Integrin-β1 resulting in a NC cell migration arrest (Testaz et al., 2001).

The initial clustering of BCCs could be mediated by Cadherin-7. Later, when motor neurons upregulate Cadherin-7, it likely mediates the adherence between motor axons and BCCs. The adherence of BCCs is then supported by N-Cadherin that is strongly upregulated once the BCC clusters are formed. This is further supported by the fact that N-Cadherin preferentially binds homophilically and is not expressed in the surrounding of BCCs or in motor neurons. Recently it was

suggested that Shh could control N-Cadherin since their expression patterns overlap spatiotemporally in the neural tube (Jarov et al., 2003). In that context it is interesting to note that Patched and Gli3 are expressed in BCCs (data not shown; Luo et al., 2006). Thus, N-Cadherin in BCCs could possibly be upregulated via notochord-derived Shh through the Patched/Smoothened/Gli pathway and “glue” BCCs to form a tight BCC cluster due to its strong adhesive force (see Figure 8).

Conclusion

The present data indicate that Semaphorin6A triggers the formation of the ventral BCC clusters at the VMEP by binding to PlexinA1 expressed on motor axons. The subsequent upregulation of cell adhesion molecules is necessary to interconnect single BCCs to a cluster and to adhere to motor axons. Different studies provide evidence of multiple mechanisms stabilizing the motor column, indicating a certain redundancy of the system. However, future studies will have to address the open questions in order to better understand the exact mechanism of BCC cluster formation and motor column stabilization.

8. Acknowledgments

I would like to thank Prof. Dr. Esther Stoeckli for giving me the chance to do my PhD on a fascinating research topic in her laboratory. Her constant support and competent guidance were valuable for my work. I always appreciated that she had an open ear for everything even though her time schedule was very busy.

Thanks to all former and present group members of the Stoeckli lab for the wonderful time in- and outside of the lab.

I would also like to thank the members of my PhD committee, Prof. Dr. Stephan Neuhauss and Prof. Dr. Martin Schwab for taking their time and providing constructive feedbacks.

I want to express my deepest gratitude to my family for their encouragements and support.

Last but not least, I want to thank Elena Domanitskaya for proofreading my thesis and supporting me in everything I'm doing. I will never forget the evenings and nights working in the lab in order to meet the deadline. Oh yeah, we were zombies the days after!

9. Curriculum Vitae

Surname:	MAUTI	First name:	Olivier
Date of birth:	20th of February 1980	Nationality:	Swiss

Education

2005 – 2008	PhD Thesis in Developmental Neurobiology, Institute of Zoology, University of Zurich
2004 – 2005	Diploma Thesis in Developmental Neurobiology, Institute of Zoology, University of Zurich Topic: "The characterization of plexins and neuropilins in the developing chicken embryo". Supervisor: Prof. Dr. Esther Stoeckli
2002 – 2004	Graduate studies in Developmental Biology and Developmental Neurobiology, University of Zurich
2000 – 2002	Undergraduate studies in Biology, University of Zurich
1996 – 2000	Gymnasium, Alte Kantonsschule Aarau, Typus C

List of publications

Mauti, O.*, Baeriswyl, T.* and Stoeckli E. T. (2008). Gene Silencing by Injection and Electroporation of dsRNA in Avian Embryos. Cold Spring Harbor Protocols. In press.

Baeriswyl, T.*, Mauti, O.* and Stoeckli, E. T. (2008). Temporal control of gene silencing by in ovo electroporation. *Methods Mol Biol* 442, 231-44.

Mauti, O., Domanitskaya, E., Andermatt, I., Sadhu, R. and Stoeckli, E. T. (2007). Semaphorin6A acts as a gate keeper between the central and the peripheral nervous system. *Neural Develop* 2, 28.

Deng, S., Hirschberg, A., Worzfeld, T., Penachioni, J. Y., Korostylev, A., Swiercz, J. M., Vodrazka, P., Mauti, O., Stoeckli, E. T., Tamagnone, L. Offermanns, S. and Kuner, R. (2007). Plexin-B2, but not Plexin-B1, critically modulates neuronal migration and patterning of the developing nervous system in vivo. *J Neurosci* 27, 6333-47.

Mauti, O., Sadhu, R., Gemayel, J., Gesemann, M. and Stoeckli, E. T. (2006). Expression patterns of plexins and neuropilins are consistent with cooperative and separate functions during neural development. *BMC Dev Biol* 6, 32.

Domanitskaya, E., Wacker, A., Mauti, O., Baeriswyl, T., Pilar, E., Bovolenta, P. and Stoeckli. E. T. Sonic hedgehog guides postcommissural axons both directly and indirectly by regulating Wnt activity. In revision.

Gemayel, J., Joset, P., Mauti, O., Sadhu, R., Baeriswyl, T., Stoeckli, E. T. and Gesemann, M. Combinatorial expression of semaphorins in chicken motor and sensory neurons suggests alternative semaphorin functions in axon growth and guidance. In revision.

Mauti, O. and Stoeckli E. T. Boundary Cap Cells – an underestimated population of cells required during neural development. In preparation.

10. References

Altman J, Bayer SA. 1982. Development of the cranial nerve ganglia and related nuclei in the rat. Adv Anat Embryol Cell Biol 74:1-90.

Altman J, Bayer SA. 1984. The development of the rat spinal cord. Adv Anat Embryol Cell Biol 85:1-164.

Aquino JB, Hjerling-Leffler J, Koltzenburg M, Edlund T, Villar MJ, Ernfors P. 2006. In vitro and in vivo differentiation of boundary cap neural crest stem cells into mature Schwann cells. Exp Neurol 198:438-449.

Arber S, Han B, Mendelsohn M, Smith M, Jessell TM, Sockanathan S. 1999. Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron 23:659-674.

Baron-Van Evercooren A, Zujovic V, Thibaud J, Deboux C, Topilko P, Charnay P. 2008. Abstracts of the American Society for Neurochemistry 39th Annual Meeting. March 1-5, 2008. San Antonio, Texas, USA. J Neurochem 104 Suppl 1:1-152.

Bear JE, Svitkina TM, Krause M, Schafer DA, Loureiro JJ, Strasser GA, Maly IV, Chaga OY, Cooper JA, Borisy GG, Gertler FB. 2002. Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. Cell 109:509-521.

Bell KM, Western PS, Sinclair AH. 2000. SOX8 expression during chick embryogenesis. Mech Dev 94:257-260.

Belmadani A, Tran PB, Ren D, Assimacopoulos S, Grove EA, Miller RJ. 2005. The chemokine stromal cell-derived factor-1 regulates the migration of sensory neuron progenitors. J Neurosci 25:3995-4003.

Bermingham JR, Jr., Shearin H, Pennington J, O'Moore J, Jaegle M, Driegen S, van Zon A, Darbas A, Ozkaynak E, Ryu EJ, Milbrandt J, Meijer D. 2006. The claw paw mutation reveals a role for Lgi4 in peripheral nerve development. Nat Neurosci 9:76-84.

Bhattacharyya A, Frank E, Ratner N, Brackenbury R. 1991. P0 is an early marker of the Schwann cell lineage in chickens. Neuron 7:831-844.

Bixby JL, Harris WA. 1991. Molecular mechanisms of axon growth and guidance. Annu Rev Cell Biol 7:117-159.

Bixby JL, Zhang R. 1990. Purified N-cadherin is a potent substrate for the rapid induction of neurite outgrowth. J Cell Biol 110:1253-1260.

Boucaut JC, Darribere T, Poole TJ, Aoyama H, Yamada KM, Thiery JP. 1984. Biologically active synthetic peptides as probes of embryonic development: a competitive peptide inhibitor of fibronectin function inhibits gastrulation in amphibian embryos and neural crest cell migration in avian embryos. J Cell Biol 99:1822-1830.

Briscoe J, Pierani A, Jessell TM, Ericson J. 2000. A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell 101:435-445.

Bron R, Vermeren M, Kokot N, Andrews W, Little GE, Mitchell KJ, Cohen J. 2007. Boundary cap cells constrain spinal motor neuron somal migration at motor exit points by a semaphorin-plexin mechanism. Neural Develop 2:21.

Bronner-Fraser M. 1986. Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. Dev Biol 115:44-55.

Bronner-Fraser M. 1994. Neural crest cell formation and migration in the developing embryo. FASEB J 8:699-706.

Carroll PM, Tsirka SE, Richards WG, Frohman MA, Strickland S. 1994. The mouse tissue plasminogen activator gene 5' flanking region directs appropriate expression in development and a seizure-enhanced response in the CNS. Development 120:3173-3183.

Cheung M, Briscoe J. 2003. Neural crest development is regulated by the transcription factor Sox9. Development 130:5681-5693.

Cheung M, Chaboissier MC, Mynett A, Hirst E, Schedl A, Briscoe J. 2005. The transcriptional control of trunk neural crest induction, survival, and delamination. Dev Cell 8:179-192.

Chu YS, Eder O, Thomas WA, Simcha I, Pincet F, Ben-Ze'ev A, Perez E, Thiery JP, Dufour S. 2006. Prototypical type I E-cadherin and type II cadherin-7 mediate very distinct adhesiveness through their extracellular domains. J Biol Chem 281:2901-2910.

David NB, Sapede D, Saint-Etienne L, Thisse C, Thisse B, Dambly-Chaudiere C, Rosa FM, Ghysen A. 2002. Molecular basis of cell migration in the fish lateral line: role of the chemokine receptor CXCR4 and of its ligand, SDF1. Proc Natl Acad Sci U S A 99:16297-16302.

Debby-Brafman A, Burstyn-Cohen T, Klar A, Kalcheim C. 1999. F-Spondin, expressed in somite regions avoided by neural crest cells, mediates inhibition of distinct somite domains to neural crest migration. Neuron 22:475-488.
Drees F, Gertler FB. 2008. Ena/VASP: proteins at the tip of the nervous system. Curr Opin Neurobiol 18:53-59.
Duband JL, Thiery JP. 1987. Distribution of laminin and collagens during avian neural crest development. Development 101:461-478.
Dutt S, Kleber M, Matasci M, Sommer L, Zimmermann DR. 2006. Versican V0 and V1 guide migratory neural crest cells. J Biol Chem 281:12123-12131.
Eickholt BJ, Mackenzie SL, Graham A, Walsh FS, Doherty P. 1999. Evidence for collapsin-1 functioning in the control of neural crest migration in both trunk and hindbrain regions. Development 126:2181-2189.
Erickson CA, Duong TD, Tosney KW. 1992. Descriptive and experimental analysis of the dispersion of neural crest cells along the dorsolateral path and their entry into ectoderm in the chick embryo. Dev Biol 151:251-272.
Ernfors P, Merlio JP, Persson H. 1992. Cells Expressing mRNA for Neurotrophins and their Receptors During Embryonic Rat Development. Eur J Neurosci 4:1140-1158.
Ernsberger U, Rohrer H. 1988. Neuronal precursor cells in chick dorsal root ganglia: differentiation and survival in vitro. Dev Biol 126:420-432.
Evans IR, Renne T, Gertler FB, Nobes CD. 2007. Ena/VASP proteins mediate repulsion from ephrin ligands. J Cell Sci 120:289-298.
Falk J, Bechara A, Fiore R, Nawabi H, Zhou H, Hoyo-Becerra C, Bozon M, Rougon G, Grumet M, Puschel AW, Sanes JR, Castellani V. 2005. Dual functional activity of semaphorin 3B is required for positioning the anterior commissure. Neuron 48:63-75.
Filbin MT, Walsh FS, Trapp BD, Pizzey JA, Tennekoon GI. 1990. Role of myelin P0 protein as a homophilic adhesion molecule. Nature 344:871-872.
Fraher JP. 1997. Axon-glial relationships in early CNS-PNS transitional zone development: an ultrastructural study. J Neurocytol 26:41-52.
Fraher JP, Dockery P, O'Donoghue O, Riedewald B, O'Leary D. 2007. Initial motor axon outgrowth from the developing central nervous system. J Anat 211:600-611.
George L, Chaverra M, Todd V, Lansford R, Lefcort F. 2007. Nociceptive sensory neurons derive from contralaterally migrating, fate-restricted neural crest cells. Nat Neurosci 10:1287-1293.
Giese KP, Martini R, Lemke G, Soriano P, Schachner M. 1992. Mouse P0 gene disruption leads to hypomyelination, abnormal expression of recognition molecules, and degeneration of myelin and axons. Cell 71:565-576.
Golding J, Shewan D, Cohen J. 1997. Maturation of the mammalian dorsal root entry zone--from entry to no entry. Trends Neurosci 20:303-308.
Golding JP, Cohen J. 1997. Border controls at the mammalian spinal cord: late-surviving neural crest boundary cap cells at dorsal root entry sites may regulate sensory afferent ingrowth and entry zone morphogenesis. Mol Cell Neurosci 9:381-396.
Goulding M, Lumsden A, Paquette AJ. 1994. Regulation of Pax-3 expression in the dermomyotome and its role in muscle development. Development 120:957-971.
Grim M, Halata Z, Franz T. 1992. Schwann cells are not required for guidance of motor nerves in the hindlimb in Splotch mutant mouse embryos. Anat Embryol (Berl) 186:311-318.
Grimsby J, Toth M, Chen K, Kumazawa T, Klaidman L, Adams JD, Karoum F, Gal J, Shih JC. 1997. Increased stress response and beta-phenylethylamine in MAOB-deficient mice. Nat Genet 17:206-210.
Guerrero I, Chiang C. 2007. A conserved mechanism of Hedgehog gradient formation by lipid modifications. Trends Cell Biol 17:1-5.
Halloran MC, Berndt JD. 2003. Current progress in neural crest cell motility and migration and future prospects for the zebrafish model system. Dev Dyn 228:497-513.
Hamburger V, Hamilton HL. 1951. A series of normal stages in the development of the chick embryo. J Morphol 88:49-92.
Herbarth B, Pingault V, Bondurand N, Kuhlbrodt K, Hermans-Borgmeyer I, Puliti A, Lemort N, Goossens M, Wegner M. 1998. Mutation of the Sry-related Sox10 gene in Dominant megacolon, a mouse model for human Hirschsprung disease. Proc Natl Acad Sci U S A 95:5161-5165.
Hjerling-Leffler J, Marmigere F, Heglind M, Cederberg A, Koltzenburg M, Enerback S, Ernfors P. 2005. The boundary cap: a source of neural crest stem cells that generate multiple sensory neuron subtypes. Development 132:2623-2632.

Horwitz A, Duggan K, Greggs R, Decker C, Buck C. 1985. The cell substrate attachment (CSAT) antigen has properties of a receptor for laminin and fibronectin. J Cell Biol 101:2134-2144.
Inoue H, Lin L, Lee X, Shao Z, Mendes S, Snodgrass-Belt P, Sweigard H, Engber T, Pepinsky B, Yang L, Beal MF, Mi S, Isacson O. 2007. Inhibition of the leucine-rich repeat protein LINGO-1 enhances survival, structure, and function of dopaminergic neurons in Parkinson's disease models. Proc Natl Acad Sci U S A 104:14430-14435.
Iozzo RV. 1998. Matrix proteoglycans: from molecular design to cellular function. Annu Rev Biochem 67:609-652.
Jarov A, Williams KP, Ling LE, Koteliansky VE, Duband JL, Fournier-Thibault C. 2003. A dual role for Sonic hedgehog in regulating adhesion and differentiation of neuroepithelial cells. Dev Biol 261:520-536.
Jessell TM. 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet 1:20-29.
Jia L, Cheng L, Raper J. 2005. Slit/Robo signaling is necessary to confine early neural crest cells to the ventral migratory pathway in the trunk. Dev Biol 282:411-421.
Jiang R, Lan Y, Norton CR, Sundberg JP, Gridley T. 1998. The Slug gene is not essential for mesoderm or neural crest development in mice. Dev Biol 198:277-285.
Jungbluth S, Koentges G, Lumsden A. 1997. Coordination of early neural tube development by BDNF/trkB. Development 124:1877-1885.
Kalcheim C, Teillet MA. 1989. Consequences of somite manipulation on the pattern of dorsal root ganglion development. Development 106:85-93.
Kapur RP. 1999. Early death of neural crest cells is responsible for total enteric aganglionosis in Sox10(Dom)/Sox10(Dom) mouse embryos. Pediatr Dev Pathol 2:559-569.
Kawasaki T, Bekku Y, Suto F, Kitsukawa T, Taniguchi M, Nagatsu I, Nagatsu T, Itoh K, Yagi T, Fujisawa H. 2002. Requirement of neuropilin 1-mediated Sema3A signals in patterning of the sympathetic nervous system. Development 129:671-680.
Kellerer S, Schreiner S, Stolt CC, Scholz S, Bosl MR, Wegner M. 2006. Replacement of the Sox10 transcription factor by Sox8 reveals incomplete functional equivalence. Development 133:2875-2886.
Kemler R. 1993. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. Trends Genet 9:317-321.
Keynes R, Tannahill D, Morgenstern DA, Johnson AR, Cook GM, Pini A. 1997. Surround repulsion of spinal sensory axons in higher vertebrate embryos. Neuron 18:889-897.
Keynes RJ, Stern CD. 1984. Segmentation in the vertebrate nervous system. Nature 310:786-789.
Kim J, Lo L, Dormand E, Anderson DJ. 2003. SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells. Neuron 38:17-31.
Klein R, Smeyne RJ, Wurst W, Long LK, Auerbach BA, Joyner AL, Barbacid M. 1993. Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. Cell 75:113-122.
Klostermann A, Lutz B, Gertler F, Behl C. 2000. The orthologous human and murine semaphorin 6A-1 proteins (SEMA6A-1/Sema6A-1) bind to the enabled/vasodilator-stimulated phosphoprotein-like protein (EVL) via a novel carboxyl-terminal zyxin-like domain. J Biol Chem 275:39647-39653.
Knabe W, Siren AL, Ehrenreich H, Kuhn HJ. 2005. Expression patterns of erythropoietin and its receptor in the developing spinal cord and dorsal root ganglia. Anat Embryol (Berl) 210:209-219.
Krull CE. 2001. Segmental organization of neural crest migration. Mech Dev 105:37-45.
Krull CE, Lansford R, Gale NW, Collazo A, Marcelle C, Yancopoulos GD, Fraser SE, Bronner-Fraser M. 1997. Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration. Curr Biol 7:571-580.
Kudo T, Kaneko M, Iwasaki H, Togayachi A, Nishihara S, Abe K, Narimatsu H. 2004. Normal embryonic and germ cell development in mice lacking alpha 1,3-fucosyltransferase IX (Fut9) which show disappearance of stage-specific embryonic antigen 1. Mol Cell Biol 24:4221-4228.
Kuhlbrodt K, Herbarth B, Sock E, Hermans-Borgmeyer I, Wegner M. 1998. Sox10, a novel transcriptional modulator in glial cells. J Neurosci 18:237-250.
Lacosta AM, Muniesa P, Ruberte J, Sarasa M, Dominguez L. 2005. Novel expression patterns of Pax3/Pax7 in early trunk neural crest and its melanocyte and non-melanocyte lineages in amniote embryos. Pigment Cell Res 18:243-251.

Lallier T, Deutzmann R, Perris R, Bronner-Fraser M. 1994. Neural crest cell interactions with laminin: structural requirements and localization of the binding site for alpha 1 beta 1 integrin. Dev Biol 162:451-464.
Landmesser L. 1978. The distribution of motoneurones supplying chick hind limb muscles. J Physiol 284:371-389.
Landolt RM, Vaughan L, Winterhalter KH, Zimmermann DR. 1995. Versican is selectively expressed in embryonic tissues that act as barriers to neural crest cell migration and axon outgrowth. Development 121:2303-2312.
Le Douarin N, Dulac C, Dupin E, Cameron-Curry P. 1991. Glial cell lineages in the neural crest. Glia 4:175-184.
Le Douarin N, Kalcheim C. 1999. The Neural Crest. Cambridge, UK: Cambridge Univ Press.
Le Douarin NM, Dupin E, Baroffio A, Dulac C. 1992. New insights into the development of neural crest derivatives. Int Rev Cytol 138:269-314.
Lee KJ, Mendelsohn M, Jessell TM. 1998. Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. Genes Dev 12:3394-3407.
Lee X, Yang Z, Shao Z, Rosenberg SS, Levesque M, Pepinsky RB, Qiu M, Miller RH, Chan JR, Mi S. 2007. NGF regulates the expression of axonal LINGO-1 to inhibit oligodendrocyte differentiation and myelination. J Neurosci 27:220-225.
Lieberam I, Agalliu D, Nagasawa T, Ericson J, Jessell TM. 2005. A Cxcl12-CXCR4 chemokine signaling pathway defines the initial trajectory of mammalian motor axons. Neuron 47:667-679.
Light W, Vernon AE, Lasorella A, Iavarone A, LaBonne C. 2005. Xenopus Id3 is required downstream of Myc for the formation of multipotent neural crest progenitor cells. Development 132:1831-1841.
Livet J, Sigrist M, Stroebel S, De Paola V, Price SR, Henderson CE, Jessell TM, Arber S. 2002. ETS gene Pea3 controls the central position and terminal arborization of specific motor neuron pools. Neuron 35:877-892.
Lo L, Dormand EL, Anderson DJ. 2005. Late-emigrating neural crest cells in the roof plate are restricted to a sensory fate by GDF7. Proc Natl Acad Sci U S A 102:7192-7197.
Luo J, Ju MJ, Redies C. 2006. Regionalized cadherin-7 expression by radial glia is regulated by Shh and Pax7 during chicken spinal cord development. Neuroscience 142:1133-1143.
Luo Y, Ferreira-Cornwell M, Baldwin H, Kostetskii I, Lenox J, Lieberman M, Radice G. 2001. Rescuing the N-cadherin knockout by cardiac-specific expression of N- or E-cadherin. Development 128:459-469.
Ma Q, Fode C, Guillemot F, Anderson DJ. 1999. Neurogenin1 and neurogenin2 control two distinct waves of neurogenesis in developing dorsal root ganglia. Genes Dev 13:1717-1728.
Mansouri A, Stoykova A, Torres M, Gruss P. 1996. Dysgenesis of cephalic neural crest derivatives in Pax7-/- mutant mice. Development 122:831-838.
Marmigere F, Ernfors P. 2007. Specification and connectivity of neuronal subtypes in the sensory lineage. Nat Rev Neurosci 8:114-127.
Maro GS, Vermeren M, Voiculescu O, Melton L, Cohen J, Charnay P, Topilko P. 2004. Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS. Nat Neurosci 7:930-938.
Mauti O, Domanitskaya E, Andermatt I, Sadhu R, Stoeckli ET. 2007. Semaphorin6A acts as a gate keeper between the central and the peripheral nervous system. Neural Develop 2:28.
Mauti O, Sadhu R, Gemayel J, Gesemann M, Stoeckli ET. 2006. Expression patterns of plexins and neuropilins are consistent with cooperative and separate functions during neural development. BMC Dev Biol 6:32.
McKeown SJ, Lee VM, Bronner-Fraser M, Newgreen DF, Farlie PG. 2005. Sox10 overexpression induces neural crest-like cells from all dorsoventral levels of the neural tube but inhibits differentiation. Dev Dyn 233:430-444.
Mi S, Lee X, Shao Z, Thill G, Ji B, Relton J, Levesque M, Allaire N, Perrin S, Sands B, Crowell T, Cate RL, McCoy JM, Pepinsky RB. 2004. LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. Nat Neurosci 7:221-228.
Mi S, Sandrock A, Miller RH. 2008. LINGO-1 and its role in CNS repair. Int J Biochem Cell Biol 40:1971-1978.
Mitchell KJ, Pinson KI, Kelly OG, Brennan J, Zupicich J, Scherz P, Leighton PA, Goodrich LV, Lu X, Avery BJ, Tate P, Dill K, Pangilinan E, Wakenight P, Tessier-Lavigne M, Skarnes WC. 2001.

Functional analysis of secreted and transmembrane proteins critical to mouse development. Nat Genet 28:241-249.
Moase CE, Trasler DG. 1990. Delayed neural crest cell emigration from Sp and Spd mouse neural tube explants. Teratology 42:171-182.
Nakagawa S, Takeichi M. 1995. Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. Development 121:1321-1332.
Nakagawa S, Takeichi M. 1998. Neural crest emigration from the neural tube depends on regulated cadherin expression. Development 125:2963-2971.
Newgreen D, Thiery JP. 1980. Fibronectin in early avian embryos: synthesis and distribution along the migration pathways of neural crest cells. Cell Tissue Res 211:269-291.
Niederländer C, Lumsden A. 1996. Late emigrating neural crest cells migrate specifically to the exit points of cranial branchiomotor nerves. Development 122:2367-2374.
Odemis V, Lamp E, Pezeshki G, Moepps B, Schilling K, Gierschik P, Littman DR, Engele J. 2005. Mice deficient in the chemokine receptor CXCR4 exhibit impaired limb innervation and myogenesis. Mol Cell Neurosci 30:494-505.
Okafuji T, Tanaka H. 2005. Expression pattern of LINGO-1 in the developing nervous system of the chick embryo. Gene Expr Patterns 6:57-62.
Oudega M, Marani E, Thomeer RT. 1992. Transient expression of stage-specific embryonic antigen-1 (CD15) in the developing dorsal rat spinal cord. Histochem J 24:869-877.
Parkinson DB, Dickinson S, Bhaskaran A, Kinsella MT, Brophy PJ, Sherman DL, Sharghi-Namini S, Duran Alonso MB, Mirsky R, Jessen KR. 2003. Regulation of the myelin gene periaxin provides evidence for Krox-20-independent myelin-related signalling in Schwann cells. Mol Cell Neurosci 23:13-27.
Pekarik V, Bourikas D, Miglino N, Joset P, Preiswerk S, Stoeckli ET. 2003. Screening for gene function in chicken embryo using RNAi and electroporation. Nat Biotechnol 21:93-96.
Perris R. 1997. The extracellular matrix in neural crest-cell migration. Trends Neurosci 20:23-31.
Persson M, Stamataki D, te Welscher P, Andersson E, Bose J, Ruther U, Ericson J, Briscoe J. 2002. Dorsal-ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity. Genes Dev 16:2865-2878.
Pietri T, Eder O, Breau MA, Topilko P, Blanche M, Brakebusch C, Fassler R, Thiery JP, Dufour S. 2004. Conditional beta1-integrin gene deletion in neural crest cells causes severe developmental alterations of the peripheral nervous system. Development 131:3871-3883.
Pindzola RR, Doller C, Silver J. 1993. Putative inhibitory extracellular matrix molecules at the dorsal root entry zone of the spinal cord during development and after root and sciatic nerve lesions. Dev Biol 156:34-48.
Pla P, Moore R, Morali OG, Grille S, Martinozzi S, Delmas V, Larue L. 2001. Cadherins in neural crest cell development and transformation. J Cell Physiol 189:121-132.
Price SR, De Marco Garcia NV, Ranscht B, Jessell TM. 2002. Regulation of motor neuron pool sorting by differential expression of type II cadherins. Cell 109:205-216.
Radice GL, Rayburn H, Matsunami H, Knudsen KA, Takeichi M, Hynes RO. 1997. Developmental defects in mouse embryos lacking N-cadherin. Dev Biol 181:64-78.
Rickmann M, Fawcett JW, Keynes RJ. 1985. The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite. J Embryol Exp Morphol 90:437-455.
Rifkin JT, Todd VJ, Anderson LW, Lefcort F. 2000. Dynamic expression of neurotrophin receptors during sensory neuron genesis and differentiation. Dev Biol 227:465-480.
Schneider-Maunoury S, Topilko P, Seitandou T, Levi G, Cohen-Tannoudji M, Pournin S, Babinet C, Charnay P. 1993. Disruption of Krox-20 results in alteration of rhombomeres 3 and 5 in the developing hindbrain. Cell 75:1199-1214.
Schneider-Schaulies J, von Brunn A, Schachner M. 1990. Recombinant peripheral myelin protein P0 confers both adhesion and neurite outgrowth-promoting properties. J Neurosci Res 27:286-297.
Semaphorin Nomenclature Committee. 1999. Unified nomenclature for the semaphorins/collapsins. Semaphorin Nomenclature Committee. Cell 97:551-552.
Serbedzija GN, McMahon AP. 1997. Analysis of neural crest cell migration in Splotch mice using a neural crest-specific LacZ reporter. Dev Biol 185:139-147.
Shibata T, Yamada K, Watanabe M, Ikenaka K, Wada K, Tanaka K, Inoue Y. 1997. Glutamate transporter GLAST is expressed in the radial glia-astrocyte lineage of developing mouse spinal cord. J Neurosci 17:9212-9219.
Sieber-Blum M. 1989. SSEA-1 is a specific marker for the spinal sensory neuron lineage in the quail embryo and in neural crest cell cultures. Dev Biol 134:362-375.

Sims TJ, Gilmore SA, Waxman SG, Klinge E. 1985. Dorsal-ventral differences in the glia limitans of the spinal cord: an ultrastructural study in developing normal and irradiated rats. J Neuropathol Exp Neurol 44:415-429.
Siren AL, Fratelli M, Brines M, Goemans C, Casagrande S, Lewczuk P, Keenan S, Gleiter C, Pasquali C, Capobianco A, Mennini T, Heumann R, Cerami A, Ehrenreich H, Ghezzi P. 2001. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. Proc Natl Acad Sci U S A 98:4044-4049.
Snider WD, Palavali V. 1990. Early axon and dendritic outgrowth of spinal accessory motor neurons studied with DiI in fixed tissues. J Comp Neurol 297:227-238.
Sock E, Schmidt K, Hermanns-Borgmeyer I, Bosl MR, Wegner M. 2001. Idiopathic weight reduction in mice deficient in the high-mobility-group transcription factor Sox8. Mol Cell Biol 21:6951-6959.
Sockanathan S, Jessell TM. 1998. Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons. Cell 94:503-514.
Southard-Smith EM, Kos L, Pavan WJ. 1998. Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. Nat Genet 18:60-64.
Stolt CC, Lommes P, Friedrich RP, Wegner M. 2004. Transcription factors Sox8 and Sox10 perform non-equivalent roles during oligodendrocyte development despite functional redundancy. Development 131:2349-2358.
Straub JA, Sholler GL, Nishi R. 2007. Embryonic sympathoblasts transiently express TrkB in vivo and proliferate in response to brain-derived neurotrophic factor in vitro. BMC Dev Biol 7:10.
Svaren J, Meijer D. 2008. The molecular machinery of myelin gene transcription in Schwann cells. Glia 56:1541-1551.
Takeichi M. 1995. Morphogenetic roles of classic cadherins. Curr Opin Cell Biol 7:619-627.
Tanabe Y, William C, Jessell TM. 1998. Specification of motor neuron identity by the MNR2 homeodomain protein. Cell 95:67-80.
Taylor J, Pesheva P, Schachner M. 1993. Influence of janusin and tenascin on growth cone behavior in vitro. J Neurosci Res 35:347-362.
Terman JR, Mao T, Pasterkamp RJ, Yu HH, Kolodkin AL. 2002. MICALs, a family of conserved flavoprotein oxidoreductases, function in plexin-mediated axonal repulsion. Cell 109:887-900.
Testaz S, Jarov A, Williams KP, Ling LE, Koteliansky VE, Fournier-Thibault C, Duband JL. 2001. Sonic hedgehog restricts adhesion and migration of neural crest cells independently of the Patched- Smoothened-Gli signaling pathway. Proc Natl Acad Sci U S A 98:12521-12526.
Thiery JP, Sleeman JP. 2006. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 7:131-142.
Topilko P, Schneider-Maunoury S, Levi G, Baron-Van Evercooren A, Chennoufi AB, Seitanidou T, Babinet C, Charnay P. 1994. Krox-20 controls myelination in the peripheral nervous system. Nature 371:796-799.
Toyofuku T, Zhang H, Kumanogoh A, Takegahara N, Yabuki M, Harada K, Hori M, Kikutani H. 2004. Guidance of myocardial patterning in cardiac development by Sema6D reverse signalling. Nat Cell Biol 6:1204-1211.
Tsai PT, Ohab JJ, Kertesz N, Groszer M, Matter C, Gao J, Liu X, Wu H, Carmichael ST. 2006. A critical role of erythropoietin receptor in neurogenesis and post-stroke recovery. J Neurosci 26:1269-1274.
Tucker GC, Aoyama H, Lipinski M, Tursz T, Thiery JP. 1984. Identical reactivity of monoclonal antibodies HNK-1 and NC-1: conservation in vertebrates on cells derived from the neural primordium and on some leukocytes. Cell Differ 14:223-230.
Tucker RP, McKay SE. 1991. The expression of tenascin by neural crest cells and glia. Development 112:1031-1039.
Valinsky JE, Le Douarin NM. 1985. Production of plasminogen activator by migrating cephalic neural crest cells. EMBO J 4:1403-1406.
Vermeren M, Maro GS, Bron R, McGonnell IM, Charnay P, Topilko P, Cohen J. 2003. Integrity of developing spinal motor columns is regulated by neural crest derivatives at motor exit points. Neuron 37:403-415.
Vitalis T, Alvarez C, Chen K, Shih JC, Gaspar P, Cases O. 2003. Developmental expression pattern of monoamine oxidases in sensory organs and neural crest derivatives. J Comp Neurol 464:392-403.
Wang HU, Chen ZF, Anderson DJ. 1998. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell 93:741-753.
Wanner IB, Guerra NK, Mahoney J, Kumar A, Wood PM, Mirsky R, Jessen KR. 2006. Role of N-cadherin in Schwann cell precursors of growing nerves. Glia 54:439-459.

Wilkinson DG, Bhatt S, Chavrier P, Bravo R, Charnay P. 1989. Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse. Nature 337:461-464.
Wissmüller S, Kosian T, Wolf M, Finzsch M, Wegner M. 2006. The high-mobility-group domain of Sox proteins interacts with DNA-binding domains of many transcription factors. Nucleic Acids Res 34:1735-1744.
Yoshida Y, Han B, Mendelsohn M, Jessell TM. 2006. PlexinA1 signaling directs the segregation of proprioceptive sensory axons in the developing spinal cord. Neuron 52:775-788.
Zhou Y, Gunput RA, Pasterkamp RJ. 2008. Semaphorin signaling: progress made and promises ahead. Trends Biochem Sci 33:161-170.
Zirlinger M, Lo L, McMahon J, McMahon AP, Anderson DJ. 2002. Transient expression of the bHLH factor neurogenin-2 marks a subpopulation of neural crest cells biased for a sensory but not a neuronal fate. Proc Natl Acad Sci U S A 99:8084-8089.
Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 393:595-599.